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CHROMATOGRAPHY OF AMINO ACIDS ON STARCH COLUMNS. SEPARATION OF PHENYLALANINE, LEUCINE, ISOLEUCINE, METHIONINE, TYROSINE, AND VALINE

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(From the Laboratories of The Rockefeller Institute for Medical Research)

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In the past few years, several methods for the chromatographic fractionation of mixtures of amino acids have been introduced by Martin and Synge and their coworkers. Chromatography of the N-acetyl amino acids on silica gel columns has been employed by Martin and Synge (1, 2), and has been further studied by Tristram (3). The separation of the free amino acids by chromatography on paper, following its introduction by Consden, Gordon, and Martin (4), has found wide use. A third method, chromatography on starch columns, has been the subject of a note by Elsdon and Synge (5), and was used by Synge (6) in qualitative experiments with partial hydrolysates of gramicidin.

It appeared that the starch column should possess characteristics rendering it the technique of choice for some types of problems. As Synge (6) pointed out, free amino acids and peptides may be chromatographed on starch, blocking of the amino group by acetylation being unnecessary. In addition, it was to be anticipated that mixtures should be fractionable in sufficient quantity with starch columns to permit subsequent examination of the components by conventional microchemical techniques. The procedure also appeared to be one which could be developed into a quantitative method for amino acid analysis.

The investigations described in this communication deal with developmental work on the chromatography of amino acids on starch columns and with specific methods for the separation and quantitative determination of phenylalanine, leucine, isoleucine, methionine, valine, and tyrosine in protein hydrolysates. Studies on the remaining common amino acids form the subject of a paper now in preparation.

In the fractionation of partial hydrolysates of gramicidin on starch columns, Synge (6) collected the effluent from the column in relatively large fractions and spot-tested each qualitatively with ninhydrin-impregnated paper. The volume of a fraction was adjusted to include substances within a given range of zone rates. Each fraction was worked up individually and its contents examined.

In the present investigations, the effluent has been collected in a regular

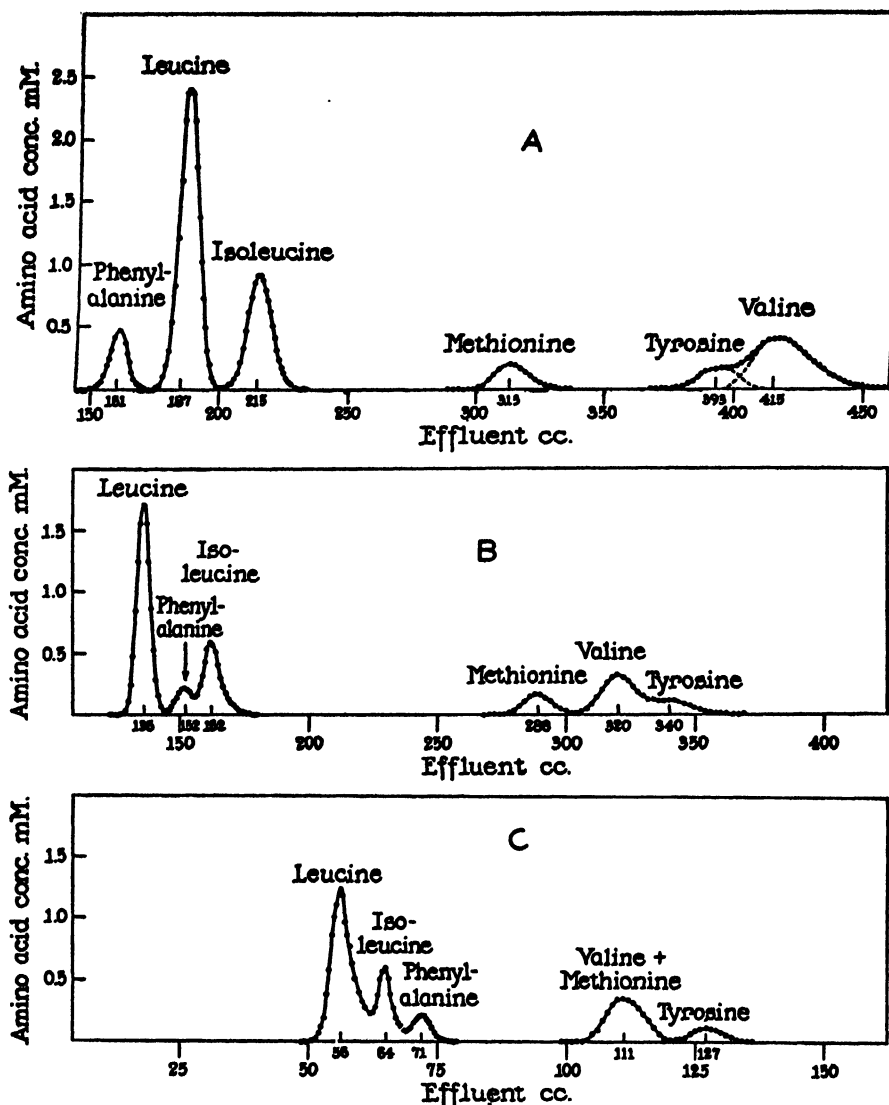


FIG. 1. Separation of amino acids from a synthetic mixture containing eighteen amino acids and NH_4Cl , corresponding in composition to an acid hydrolysate of β -lactoglobulin. Amino acid concentrations are given in leucine equivalents (11). A, solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water. Column, 52 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 30 cm. Sample, 20 mg. of synthetic mixture. B, solvent, *n*-butanol-15 per cent water. Column, 55 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 29 cm. Sample, 10 mg. of synthetic mixture. C, solvent, *n*-butanol-17 per cent 0.57 *N* HCl. Column, 55 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 28 cm. Sample, 5 mg. of synthetic mixture.

series of small fractions of known volume, and the fractions have been analyzed quantitatively rather than qualitatively. The data thus obtained permit the construction of effluent concentration curves which reveal the detailed behavior and the full resolving power of the column. The effluent fractions have been collected with the aid of a specially constructed, fully automatic fraction-collecting machine, and the fractions have been analyzed quantitatively for amino acids by means of a photometric ninhydrin method developed for this purpose.

Effluent concentration curves showing the behavior of six amino acids in three solvents are given in Fig. 1. These amino acids are the first to emerge from a starch column when the sample fractionated consists of a known mixture of eighteen amino acids and ammonium chloride made up to simulate the composition of an acid hydrolysate of β -lactoglobulin (cf. data of Brand *et al.* (7)). The procedure employed to obtain curves of the type shown in Fig. 1 is given in the experimental section, followed by a discussion of the influence of variables on the process and by a summary of the results obtained with protein hydrolysates.

Procedure

Preparation of Starch Column—The chromatograph tubes used in these experiments were of the Zechmeister-Cholnoky type with ground joints and sintered glass plates.¹ The following directions are for the preparation of a 30 cm. starch column in a tube of 0.9 cm. inner diameter and 40 cm. in length. Direct proportionality factors can be used for columns of other dimensions. The columns were prepared and run in an air-conditioned room at $25^\circ \pm 0.5^\circ$. There have been no indications that a constant temperature room is essential. Recent experiments have indicated that satisfactory results can be obtained at room temperatures of $25^\circ \pm 5^\circ$.

The starch employed in this investigation was potato starch powder manufactured by Morningstar Nicol, Inc.² Different batches procured from the same manufacturer have given fully reproducible results. In order to obtain the correct tightness of column packing, the water content of the starch at the time the column is prepared must be carefully controlled. The moisture content of the air-dried product is determined by drying a sample to constant weight at 110° at atmospheric pressure. The moisture content of starch may vary with the atmospheric humidity, and should be checked periodically. An air-dried sample corresponding to 13.4 gm. of

¹ Purchased from the Scientific Glass Apparatus Company, Bloomfield, New Jersey, catalogue No. J-1664. A length of 40 cm. above the sintered plate must be specified for use with starch columns 30 cm. in height.

² Manufactured from white potatoes by Morningstar Nicol, Inc., New York, and purchased from Amend Drug and Chemical Company, Inc., New York.

anhydrous starch is weighed out. For the starch used in these experiments, the sample was suspended in 25 cc. of dry butanol in which enough water had been dissolved to bring the total water present to 30 per cent of the dry weight of the starch. For starch containing 20 per cent moisture, 16.8 gm. were weighed out and suspended in butanol containing 0.7 cc. of water. The starch is stirred thoroughly with a glass rod until a uniform suspension free from lumps is obtained. During this time the starch adsorbs most of the water present in the butanol. The suspension is poured into the upright chromatograph tube through a funnel possessing a tip bent to touch the side of the tube. In this manner, the suspension flows down the side of the tube without incorporation of air bubbles. For chromatograph tubes up to 2 cm. in diameter, a 20 cm. extension of glass tubing of the same diameter is attached to the top of the tube during the pouring process. This extension is necessary to accommodate the full volume of the slurry. The connection is secured glass to glass with rubber tubing. For tubes possessing a diameter larger than 2 cm., the columns can be packed in portions without an extension by first pouring two-thirds of the slurry and adding the remainder after the first portion has settled to constant height.

After the suspension has been poured into the tube, the column is placed under an air pressure of 5 to 7 cm. of mercury.³ The starch settles slowly over a period of 1 to 3 hours. If the moisture content of the starch has been correctly adjusted, a sharp settling line can be seen to move steadily up the tube when the column is illuminated from behind with a strong light. When the starch has packed to a constant height, the extension tube, if one was used, is removed and the butanol remaining on the surface of the column is withdrawn with a pipette attached to a rubber bulb. The solvent with which the chromatogram is to be run is added carefully to the top of the column without disturbing the surface of the starch. The tube is filled to within 5 cm. of the top. A 125 cc. separatory funnel filled with solvent is attached to the column by a micro rubber stopper (Arthur H. Thomas Company, catalogue No. 8823-A) through which the constricted tip extends about 2 cm. An air lock is thus formed between the solvent and the stopper in order to avoid contact of the liquid with the rubber. A pressure of 8 cm. of mercury is applied to the top of the separatory funnel and main-

³ For routine use, reducing valves are installed on the compressed air lines. Air, filtered through a $\frac{1}{4}$ inch Logan aridifier (Crane and Company, 47-30 29th Street, Long Island City, New York) is drawn at 70 pounds pressure through a reducing valve type R-79 (Linde Air Products Company, 205 East 42nd Street, New York). The outlet gage on the valve is replaced by a test gage reading from 0 to 25 cm. of mercury, $2\frac{1}{4}$ inches in diameter, procured from the Factory Products Company, 161 Meserole Avenue, Brooklyn 22. A T-tube with a rubber tube and screw clamp is placed in the line to the chromatograph tube to provide a constant "bleeder."

tained until about 50 cc. of solvent have passed through the column. This procedure, which usually requires about 36 hours, permits the starch to adsorb enough water to become equilibrated with the wet organic solvent. The resulting swelling of the starch may cause the surface of the column to rise 1 to 2 cm. If butanol-benzyl alcohol solvent mixtures have been employed, the column will gradually become translucent during the equilibration process.

After the starch columns have become fully equilibrated, they are operated under a pressure of 15 cm. of mercury. Pressure in excess of 8 cm. during the equilibration period, or of 15 cm. during operation, may cause the generation of air bubbles in the lower half of the column, and, therefore, should not be used. The presence of a small number of air bubbles within a few cm. of the sintered plate is frequently noted, and does not interfere with the results.

The degree of tightness of the column packing must be checked by measurement of the flow rate after equilibration with butanol-water or butanol-benzyl alcohol-water. A satisfactory column, 0.9 cm. in diameter and 30 cm. in height, should possess a flow rate of 1.25 to 1.50 cc. per hour at 15 cm. of mercury pressure (2.0 to 2.4 cc. per hour per sq. cm. of cross-sectional area on a column 30 cm. in height). Preparations of potato starch from different sources may vary in average particle size, which can affect the amount of water which should be present when the column is poured. In general, it can be suggested that if too fast or too slow a flow is obtained, the water content at the time of pouring should be diminished or increased to establish the optimum conditions for the preparation being used.

The surface of the column must be firmly packed before the sample is added. The solvent is removed to within 1 to 2 mm. of the starch. The remaining liquid is driven down at 7 cm. pressure until it clears the surface. The pressure is maintained for several minutes until the starch has fallen 1 to 2 mm. If any scum from the solvents has collected on the surface of the column, the top 1 mm. of starch should be removed with a silver spatula. The surface is tamped and leveled with a flat tipped 6 mm. glass rod. Air pressure is reapplied until the surface is fairly dry as evidenced by a frosty appearance. The surface is tamped again with the glass rod, and the process repeated until a firm, smooth surface which does not cling to the rod is obtained. For columns larger than 0.9 cm. in diameter, the preliminary packing of the surface can be performed with a spatula and the final smoothing of the surface with a glass or stainless steel plunger possessing a diameter a few mm. less than that of the chromatograph tube. Care must be taken while preparing the surface not to continue the air pressure for too long a period. If this is done, the starch will pull away from the

walls of the tube and air bubbles will be seen below the surface. If the bubbles extend only a few mm., the column still gives satisfactory results. If the bubbles extend down several cm., the column should be discarded.

Except when strongly acidic solvents are being used, starch columns require treatment with 8-hydroxyquinoline to remove traces of interfering metal ions. For columns 0.9 cm. in diameter, a solution of 25 mg. of 8-hydroxyquinoline (Merck) in 2.5 cc. of the solvent used on the column is added to the tube and driven into the starch at 15 cm. pressure. When this solution has just cleared the surface, fresh solvent is added and run in until the yellowish green band of hydroxyquinoline is at least 5 cm. below the surface.

The column is then ready for the addition of the sample. If not used immediately, the columns can be allowed to drip under gravity or can be kept for at least several weeks with the solvent and separatory funnel (stop-cock closed) on the top and with the tip immersed in a test-tube filled with solvent. At the end of the experiment, the starch is conveniently removed from the narrow chromatograph tubes by a jet of water from stiff tubing (Tygon). Columns run with the solvents described in this communication should not be used more than once. The exception to this statement is the case in which the mixture applied to the column contains only the amino acids shown in Fig. 1 (or tryptophan) and is free of acids or neutral salts.

Addition of Amino Acid Sample to Column—For the curves shown in Fig. 1, an amino acid mixture⁴ simulating the composition of an acid hydrolysate of β -lactoglobulin (7) was employed. A total of about 1 gm. of amino acids was dissolved in 1.5 cc. of approximately 6 N HCl and made up to a volume of 10 cc. with water. Methionine and cysteine were not included in this stock mixture, although subsequent experiments have shown that methionine may be incorporated without risk of deterioration on storage of the solution at 3°. Immediately before an experiment, the appropriate quantities of methionine and cysteine hydrochloride were made to a volume of 10 cc. with 0.1 N HCl. To a 10 cc. volumetric flask, 0.5 cc. samples of each of the aqueous solutions referred to above were added and the mixture was

⁴ All the amino acids employed were checked for correct elementary analysis (carbon, hydrogen, and nitrogen). Specific rotations were measured on the L-amino acids. The following preparations were used: DL-leucine, DL-isoleucine, DL-methionine, DL-valine, DL-aspartic acid, DL-threonine, glycine, L-arginine hydrochloride, L-cysteine hydrochloride, and L-cystine (all Merck); L-tyrosine and L-glutamic acid (Corn Products, recrystallized); L-proline from gelatin (8); L-alanine, L-serine, L-leucine, L-phenylalanine, L-histidine \cdot HCl \cdot H₂O, and L-lysine dihydrochloride prepared from protein hydrolysates (9). We are indebted to Dr. Erwin Brand for samples of L-tryptophan and L-valine, to Dr. Karl Folkers of Merck and Company, Inc., for a sample of L-methionine, and to Dr. E. E. Howe of the same company for the DL-leucine and DL-isoleucine.

made to volume with *dry* butanol or 1:1 butanol-benzyl alcohol. If difficulty is encountered in dissolving the amino acid mixture upon shaking, it may be necessary to add 0.2 to 0.4 cc. of ethanol and 1 or 2 drops of 6 N HCl before the solution is diluted to volume. For a column 0.9 cm. in diameter 0.5 cc.⁵ of the resulting solution, corresponding to about 2.5 mg. of total amino acids, is added to the top of the column, care being taken not to disturb the surface of the starch. The experiments illustrated in Fig. 1 were run on a column 1.9 cm. in diameter with a 2 cc. sample of the amino acid solution.

Air pressure (15 cm.) is applied and the sample driven into the column. After the liquid has reached the level of the starch, 0.2 cc. of solvent is added to rinse down the walls of the tube. The wash solution is forced into the column under pressure. The washing operation is carried out three times. Solvent is added over the column, the reservoir connected, and air pressure of 15 cm. is applied. Measurement of the effluent volumes recorded in Figs. 1, 3, and 4 was started at this time. A graduate is placed under the column and an appropriate fore fraction is usually taken before the column is placed on the fraction-collecting machine. For the first run on an unknown sample, a fore fraction is not taken and the curve is checked from the beginning for possible peaks ahead of phenylalanine and leucine.

The developmental work to establish the positions of the peaks with different solvents was done with synthetic mixtures containing one to six components. A mixture containing 50 to 150 mg. of each component was made to a volume of 25 cc. in 0.5 N HCl and 1 cc. diluted to 10 cc. with the dry organic solvent. Addition of the amino acids in different millimolar concentrations permits identification of the peaks by height and integration.

It will be noted that stock amino acid mixtures are stored in aqueous HCl solution and made to volume with the desired organic solvent immediately before use. The excess alcoholic solution is discarded. No alteration in the composition of the aqueous mixtures has been noted when they are stored at 3° for periods up to several months. A progressive decrease in the amino acid content of mixtures of amino acids stored in the acidic alcoholic solutions has been observed. Concomitantly with the decrease in free amino acid content of such solutions, the appearance has been noted of material which yields a fast moving zone on the column and gives a positive color reaction with ninhydrin. The behavior on the column of such altered amino acid solutions has been simulated by the addition, to fresh amino acid solutions, of an amino acid ester, such as phenylalanine ethyl ester hydrochloride. Some of the amino acids are esterified, therefore, upon prolonged standing in the acidic alcohol-water solvent mixtures. *The

⁵ For quantitative work each pipette used was calibrated for delivery with the given organic solvent mixture.

brief period of contact while the solution is being added to the column is insufficient to cause losses. Nearly quantitative recoveries are obtained even when an acidic solvent is employed in the development of the chromatogram (butanol-17 per cent 0.57 N HCl, Fig.1).

In general, the amino acids are added to a column as their hydrochlorides in order to render the amino acids more readily soluble in the organic solvents. Of the six amino acids included in Fig. 1, all but tyrosine can be brought into solution in neutral butanol-water or butanol-benzyl alcohol-water. Chromatography of the neutral amino acid samples yields effluent curves indistinguishable from those obtained when the hydrochlorides of the amino acids are added to the column. The HCl, when included in the sample, is stripped away by the first few cm. of the starch. The fate of HCl can be visualized by incorporating a trace of methyl orange in the solvent. The HCl gives a zone which moves at about half the rate of valine and which fades out after traveling about 3 cm. as a result of gradual neutralization by the traces of basic groups present in the potato starch.

For the majority of the present experiments with protein hydrolysates, the proteins were hydrolyzed for 16 hours with 10 times their weight of boiling 6 N HCl. The most recent analyses have been carried out on hydrolysates prepared by dissolving the protein in a minimum volume of water or dilute HCl and adding 200 times the sample weight of 6 N HCl⁶ twice distilled in glass. The mixture was refluxed for 16 hours on an oil bath, the oil level being kept below that of the contents of the flask. In both instances, the excess HCl was removed by repeated concentrations under reduced pressure. The hydrolysate was not filtered but was washed into a volumetric flask with small portions of water and made up to a volume corresponding to about 50 mg. of the original protein per cc. For chromatographic analysis, aliquots of this solution were diluted (0.5 cc. to 5 cc.) with the organic solvent and applied to the column in the manner outlined for the synthetic mixtures. The quantity of protein to which a given amount of hydrolysate corresponded was determined by micro-Kjeldahl nitrogen analyses on the protein and on the hydrolysate. The analyses indicated on the average about 2 per cent manipulative loss during the concentration procedure. A nitrogen analysis was also performed upon a solution obtained after 50 cc. of 6 N HCl, containing no protein, had been refluxed for 16 hours and concentrated to a volume of 5 cc. The nitrogen contributed by NH₃ in the HCl was negligible.

Collection of Effluent Fractions—In the early stages of this work, the

⁶ Dr. C. F. Jacobsen has discussed with us his unpublished experiments which have demonstrated the advantages of carrying out the hydrolysis in relatively dilute solution with HCl which is as free as possible from heavy metal impurities. Humin formation is reduced under these conditions.

effluent fractions were collected manually. The performance of a large number of experiments of the kind illustrated in Fig. 1 became a practicable laboratory procedure only after the construction of a fully automatic fraction-collecting machine, drawings of which are given in Fig. 2.^{7,8}

The machine is designed to collect the effluent from the chromatogram in successive fractions of known volume. The fractions are accumulated in tubes held in the large circular test-tube rack which accommodates 80 test-tubes in each of four concentric circles. The delivery tip of the chromatograph tube makes contact with the bottom of the enlarged part of the funnel (*A*) which is mounted in the phototube housing (*B*). The funnel is a Pyrex Gooch crucible holder 32×160 mm. (Corning Glass Works, catalogue No. 9480), the tip of which has been constricted. Each drop falling from the tip of the funnel intercepts a light beam which is focused through a circular aperture $\frac{1}{8}$ inch in diameter upon a phototube (RCA No. 921). The tip of the funnel must be mounted above the aperture at such a height that the emerging drops intercept the light beam just as they leave the tip. If the tip is too low, hanging drops will register; if too high, the resulting free falling drop will pass through the light beam too rapidly to cause an interruption of the beam of sufficient duration to register the drop. The height of the funnel can be adjusted by sliding it in the clamp (*C*). The glass shield (*D*) minimizes evaporation from the drop.

The change in current of the phototube circuit occasioned by the falling drop is amplified through the relay (*E*) and fed into the automatic reset impulse counter (*F*). The phototube, lens, light source and relay system are available from the Langevin Corporation, 37 West 65th Street, New York (model PR-411-A), or from the Ripley Company, Inc., Deep River, Connecticut (modified 70 series, model No. 8382). The housing and mountings were constructed of bakelite. The impulse counter was purchased from the Eagle Signal Corporation, Moline, Illinois (type HZ-50A6; housing HN-84; wiring arrangement No. 1). The impulse counter can be set to record any number from 1 to 400 drops. After the preset number of drops has been registered, the counter resets itself to zero and turns on the motor (*G*) (110 volt, 60 cycle A.C., 1/20 horse power, 1725 R.P.M., 10:1 gear reduction; Boston Gear Works, North Quincy, Massachusetts, catalogue No. MB-5810-S⁹). By means of a belt drive to the wheel (*H*), the motor turns the

⁷ This machine was constructed with the aid of Mr. Joseph Blum of the Instrument Shop of the Institute. A photograph of the fraction collector is included in another report (10).

⁸ A fraction collector based on this design is available commercially from the Technicon Company, 215 East 149th Street, New York 51. Convenient 50 tube aluminum racks with cellophane covers for handling the 18×150 mm. test-tubes are also available from this source.

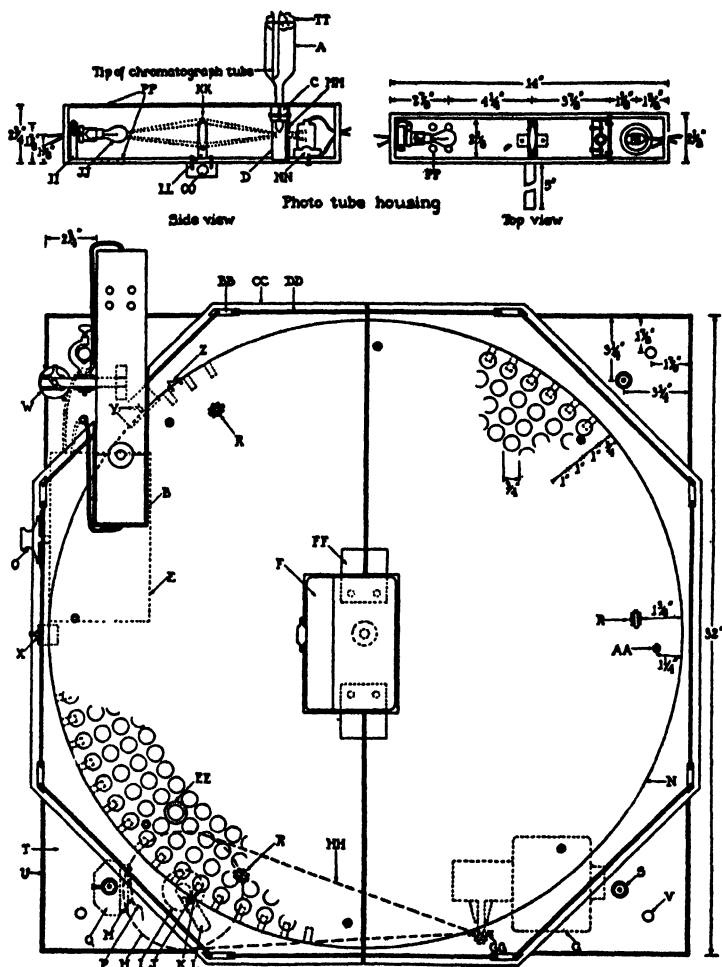
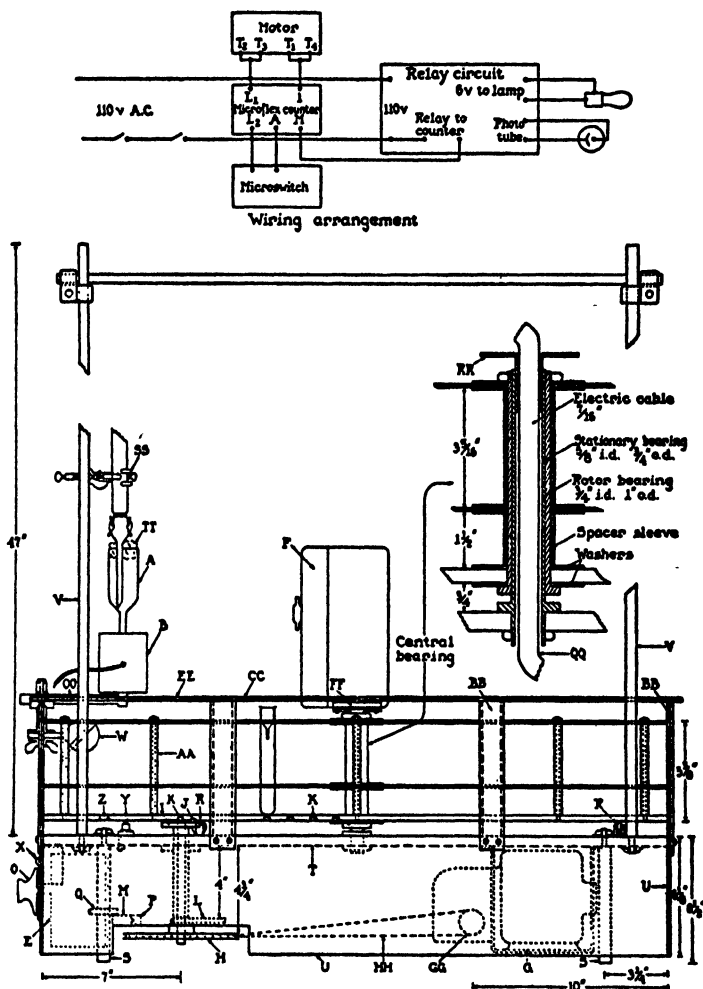


FIG. 2. A. Automatic fraction collector for chromatographic analysis, top view

FIGS. 2, A AND B. A, funnel; B, phototube housing (base and ends $\frac{1}{2}$ inch bakelite, sides $\frac{3}{16}$ inch, top $\frac{3}{32}$ inch, split into two sections at the funnel); C, split bakelite clamp for funnel ($\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2}$ inches, $\frac{5}{16}$ inch hole, thumb-screw adjustment); D, glass shield (1.8 cm. tubing, 2 inches long); E, relay held to base of machine by brackets; F, impulse counter; G, motor; H, 6 inch diameter pulley; I, brass wheel, 2 inches in diameter, $\frac{3}{16}$ inch thick; J, brass engaging pin (centered $\frac{1}{2}$ inch from center of shaft, $\frac{1}{2}$ inch high, $\frac{1}{2}$ inch in diameter, with freely turning collar $\frac{19}{64}$ inch outer diameter); K, slots to accommodate engaging pin ($\frac{5}{16}$ inch wide, $\frac{5}{16}$ inch high, 1 inch deep, centers 4.5° apart, directly under the holes of the rack); L, arm on pulley shaft to actuate micro switch; M, micro switch (type 132-R, brown top); N, test-tube rack with four concentric circles each possessing eighty $\frac{1}{2}$ inch holes (base $\frac{1}{2}$ inch bakelite, rack $\frac{3}{32}$ inch); O, sensitivity dial for relay circuit, graduated 0 to 100 over 270° , under spring



tension to hold the knob in a set position; *P*, brass spring arm over micro switch; *Q*, aluminum plate to which microswitch is attached, held on leg of base by set screw; *R*, rollers ($\frac{1}{4}$ inch high, wheels $\frac{1}{4}$ inch diameter, $\frac{1}{4}$ inch wide); *S*, legs, $\frac{1}{4}$ inch aluminum rod turned down to $\frac{5}{16}$ inch at the top, threaded for bolting through base; *T*, base plate, bakelite, $32 \times 32 \times \frac{1}{4}$ inches; *U*, side panels, $\frac{3}{32}$ inch bakelite (section cut out for 6 inch pulley on right side); *V*, $\frac{1}{4}$ inch Flexaframe rods, uprights turned down at the bottom to $\frac{5}{16}$ inch, threaded for bolting through the base, with cross-braces at the top; *W*, clamp, castaloy, Fisher catalogue No. 5-764; *X*, on-off toggle switch; *Y*, toggle switch for automatic cut off; *Z*, beveled arm to throw automatic cut off switch, arm screwed to base of rack between slots; *AA*, one of eight spacers for rack, $\frac{1}{4}$ inch center rod, $\frac{5}{16}$ inch spacing sleeves; *BB*, slotted brass supports for side panels of the cover; *CC*,

wheel (*I*) which is adjacent to the rack. The upright pin (*J*) on this wheel engages one of the slots (*K*) in the base of the circular test-tube rack and moves the rack one-eightieth of a turn. A new tube is thus brought under the column. The arm (*L*) on the shaft makes contact with the micro switch (*M*) and turns off the motor after the wheels (*H* and *I*) have made one revolution. Since the counter mechanism requires that the load circuit be closed for nearly 1 second to allow time for complete resetting, it is necessary for the gear ratio to give not more than one revolution of *H* per second. The moving parts of the rack are machined from brass. The rack revolves on a sleeve bearing at the center and is supported by three symmetrically placed rollers (*R*) mounted on the base.

The fraction collector is designed for continuous duty. The counter and the relay can be replaced readily in case of failure. An extra one of each of these items should be kept on hand as replacement parts. The relay is mounted open to the air (not in a closed housing) under the base of the machine to avoid overheating. The light bulb is changed routinely after each month of continuous duty. In the installation of the photoelectric counter, it is necessary to keep the low resistance leads to the phototube short enough to bring the circuit into counting balance when most of the variable resistance (*O*) is in the circuit. The relay should close when the sensitivity dial (*O*) is turned to about 70. Accurate focusing of the light beam on the aperture is essential for this result and the aperture in the diaphragm may have to be enlarged if the light intensity striking the phototube is too low. The adjustment of the tip should be such that drops still register when the sensitivity dial is near zero. For maximum stability during operation, the dial is set about midway between the point at which the counter almost fails to register drops and the dial setting at which the counter circuit is permanently closed. The relay is designed to give an impulse of about 0.5 second duration to the counter upon interruption of the light beam for 0.01 second. The maximum counting rate with this machine is, therefore, about 2 drops per second.

$\frac{1}{4}$ inch Lucite cover, split in center, cut out around phototube housing; *DD*, 1/16 inch Lucite side panels, 7 $\frac{1}{4}$ inches high; *EE*, 1 $\frac{1}{2}$ inch hole in cover, centered over third row of rack; *FF*, center supports for cover, attached to base of counter; *GG*, 1 inch diameter pulley; *HH*, 3/16 inch leather belt; *II*, split bakelite clamp for lamp socket; *JJ*, lamp, General Electric 87, 6 to 8 volts, 15 candle power, mounted with the filament vertical; *KK*, lens, 1 $\frac{1}{2}$ inches in diameter, 2 inch focal length; *LL*, grooved lens holder of bakelite, providing a firm grip on the lens; *MM*, removable diaphragm of 3/32 inch bakelite, supporting clamp *C*, sliding into grooves in side walls of housing, with $\frac{1}{4}$ inch diameter aperture for light beam; *NN*, phototube, RCA 921; *OO*, $\frac{1}{4} \times 5$ inch supporting rod for housing; *PP*, ventilating holes in housing; *QQ*, six wire electric cable to counter; *RR*, base plate for counter, with hollow central shaft fitting tightly into stationary bearing; *SS*, clamp, castaloy, Fisher catalogue No. 5-743; *TT*, cotton packing around stem of chromatograph tube.

In the design of the fraction collector, operation on the drop-counting principle was chosen to provide rigorous control of the sample size. Experience with the machine has demonstrated that, with a dependable drop counter of the type recommended, considerable convenience and accuracy are afforded by this method of operation. For chromatographic work in general, fraction collectors operated on a time basis would probably not give as uniform fraction size, since flow rates through a column are seldom strictly constant, even when constant pressure devices are employed.

The base of the machine and the circular rack should be made of materials which do not warp and are not damaged by organic solvents or dilute acids. The rack should be of material which will not scratch photometer tubes. Bakelite has been used in the present instrument, although investigations performed recently and to be described in a forthcoming paper have revealed that this material suffers from the disadvantage that it contains ammonia or other volatile nitrogenous compounds. In chromatographic experiments with the amino acids not included in the present work, acidic solvents, such as 2:1 *n*-propanol-0.5 *N* HCl, have proved extremely useful. Such solvents absorb ammonia very readily. Since as little as 0.1 γ of NH_3 per cc. is detected by the ninhydrin method, the NH_3 content of the bakelite has been troublesome. It has been possible to obtain satisfactory operation by painting the bakelite surfaces of the machine and of the phototube housing with a 2 per cent alcoholic solution of citric acid. Sheets with a dull finish take this treatment better than polished bakelite. Nevertheless, in building the fraction collector, it would be preferable to use materials which do not liberate ammonia. Provided the manufacturers cannot furnish bakelite meeting these specifications, a possibility currently being investigated, other materials might be substituted. Any material employed should first be tested by suspending a sample of it over a few cc. of 2:1 propanol-0.5 *N* HCl in a closed vessel for 24 hours. Analysis of neutralized 0.5 cc. aliquots of the solvent by the ninhydrin method should reveal the presence in the material of any volatile nitrogenous compounds which might be a source of interference. The Lucite cover is not resistant to butanol or benzyl alcohol and it may prove preferable to make covers from other materials. A sliding addition to the cover can also be made to fill in the space beneath the phototube housing.

In a chromatographic experiment, the starch column, to which the sample of amino acids has already been added, is mounted on the fraction collector. The solvent reservoir is attached, and the requisite air pressure applied. The stop-cock on the reservoir is clamped firmly in position to prevent it from loosening under pressure. With columns 0.9 cm. in diameter, 0.5 cc. fractions are collected. In general, in order to realize the full resolving power of the column, the size of the fractions collected should be

small enough to yield at least ten points on each amino acid peak. The number of drops required for a given volume is readily determined from the weight of the liquid delivered in 10 drops from the tip, together with the density of the solvent. For small volumes, a tip which delivers 20 to 40 drops per 0.5 cc. is convenient. The size of drop delivered is not significantly changed by a 2-fold alteration in the rate of flow. The number of drops per cc. from a given tip is constant to better than 1 per cent for a given solvent over a temperature range of several degrees and is unaffected by dilute concentrations of solute in the effluent. The drop size can be increased to a maximum of about 20 drops per cc. with a fairly blunt tip of 8 mm. outer diameter. If water is being used, rather than an organic solvent mixture, a beveled tip or a constricted tip of wider bore may be required for even flow.

The machine is designed to hold 80 lipless soft glass test-tubes, 18×150 mm. (Arthur H. Thomas Company, catalogue No. 9446). For analytical experiments with columns 0.9 cm. in diameter, much time is saved by employing tubes that have been calibrated for use in the spectrophotometer (11). Since the whole fraction is analyzed, evaporation of some of the solvent introduces no error. If larger columns are being run for preparative purposes, uncalibrated tubes are employed and aliquots pipetted for analysis. In this case, evaporation can frequently be neglected, since with butanol it amounts to only about 50 mg. per tube in 18 hours. If desired, evaporation from the tube can be reduced almost to zero by placing a small funnel in each tube, as illustrated in the receiver in Fig. 2, *B*. The funnels can be made from 15×125 mm. Pyrex test-tubes with rims. In the collection of large fractions, the splashing which frequently occurs as the drops strike the funnels can be eliminated by placing a small pad of glass wool in each funnel.

The tubes are permanently numbered with a serial number and a set number and stored in sets of 100 to 200 tubes in soft aluminum racks,⁸ protected from dust by covers. The filled tubes are removed from the fraction collector each day and replaced by empty receivers. The fractions are stoppered with corks until ready for analysis. A typical run for the six peaks shown in Fig. 1 requires continuous operation of the machine for about 4 days. Under these conditions the automatic cut-off arm (*Z*) is removed from the base of the rack. If it is desired to have the machine turn off after filling a prescribed number of tubes, this arm can be placed in position. As many as four columns can be run on one machine at the same time. For example, a second column can be mounted on the right side over the second or third row of receivers and the pressure adjusted to give about the same rate of flow as that of the column feeding into the photoelectric counter. The tip, with cotton packing around the stem, is protected by

a glass shield fitting into an opening in the cover (*EE*, Fig. 2). For analytical experiments with columns 0.9 cm. in diameter, this arrangement can be satisfactory, since the exact fraction size does not enter into the integration of the curves. However, if the fraction size is not accurately known, the estimate of the position of a peak in terms of cc. on the abscissa of the effluent curve may be in error. If the second row experiment is a duplicate of one that has been run on the counter, the peaks can usually be identified by cross-reference to the sequence of peaks in the first determination. Columns on the inner rows can also be used for exploratory qualitative experiments.

Analysis of Effluent Fractions—When columns 0.9 cm. in diameter are employed, entire 0.5 cc. effluent fractions are analyzed by the photometric ninhydrin method outlined in the following paper (11). With larger columns, 0.1 to 0.5 cc. aliquots of the effluent fractions are pipetted for analysis. The convenience of eliminating the pipetting step points to the desirability of employing columns 0.9 cm. in diameter for experiments being run for analytical purposes.

In the plotting and integration of the curves, it is important that the baseline be correctly chosen. With each set of 50 fractions taken for analysis, six to eight fractions well ahead of or behind the peaks should be included. These fractions determine the blank reading of the effluent from the column. The positive fractions are read against the average tube of the blank series as zero.

The curves are integrated by the addition of the analytical values for the points on a given peak. In determining the mg. of sample placed on the column, calibration factors for the delivery of the pipettes with aqueous and alcoholic solutions are included (*cf.* (11), Table V).

When two or more amino acids are incompletely separated from one another, as in the case of tyrosine and valine in butanol-benzyl alcohol (Fig. 1), the amounts of each can be calculated if the overlap does not extend as far as the peak points. The calculation depends upon two experimentally observed facts; namely, (a) the height of a given peak is proportional to the amount of amino acid represented by the peak, and (b) for a given column, the height of a peak of unit area decreases approximately linearly with increasing effluent volumes. In general, the calculation is made as follows:

A_1 , A_2 , and A_3 = quantities in micromoles of amino acid in the first, second, and third peaks

P_1 , P_2 , and P_3 = corrected height of each peak in millimolar concentration from Tables I and III of the following paper (11)

V_1 , V_2 , and V_3 = effluent volumes at which the peaks emerge

F_1 , F_2 , and F_3 = color yields for amino acids A_1 , A_2 , and A_3

T = micromoles (leucine equivalents) obtained by integration of the combined peaks, as in Table V (11), not corrected for color yield

$$A_1 = \frac{P_1 T}{F_1 \left(P_1 + \frac{V_2}{V_1} P_2 + \frac{V_2}{V_1} P_3 \right)}$$

$$A_2 = \frac{\frac{V_2}{V_1} P_2 T}{F_2 \left(P_1 + \frac{V_2}{V_1} P_2 + \frac{V_2}{V_1} P_3 \right)}$$

$$A_3 = \frac{\frac{V_2}{V_1} P_3 T}{F_3 \left(P_1 + \frac{V_2}{V_1} P_2 + \frac{V_2}{V_1} P_3 \right)}$$

The recoveries of tyrosine and valine summarized in Table I have been calculated by this method by using only the terms involving P_1 and P_2 for a double peak. The calculation gives results within ± 5 per cent of the theoretical recoveries in this instance. In order to apply these equations, it is necessary to have several analytical points near the peak of the curve. The recoveries are less satisfactory when the peaks are rising so rapidly that the maximum concentrations are not well defined. It is also necessary that the fraction representing the peak of each curve should contain only one amino acid. The decision as to whether the peaks are sufficiently separated to permit valid calculation can generally be made by inspection of the curves. With unknown mixtures, the applicability of the calculation should be checked by an experiment with a known mixture containing tyrosine and valine in approximately the same proportions found in the unknown. The use of partially separated peaks for analytical purposes is to be avoided whenever possible by the choice of a more favorable solvent mixture. The calculations given above have proved useful, however, for preliminary approximations, as well as in securing the data on tyrosine and valine given in Tables I, II, and III.

In some instances with larger chromatograms, it is convenient to be able to spot-test the fractions with ninhydrin-impregnated paper in order to locate the positions of major peaks and valleys. Strips of filter paper can be impregnated with a solution of 100 mg. of ninhydrin in 10 cc. of *n*-propanol and 20 cc. of 0.2 M citrate buffer, pH 5. The air-dried paper can be stored for a week or more in the dark without significant deterioration. After the application of a small drop from each fraction, the paper is warmed to 80° for development of the blue color. With butanol-benzyl alcohol, it is desirable to place a drop of water on the paper before adding the organic solvent.

Solvent Mixtures^a—The solvent which has proved most useful for the separation of the first six amino acids (Fig. 1) contains *n*-butanol, benzyl alcohol, and water. Consden, Gordon, and Martin suggested the use of this combination of alcohols in paper chromatography (4). On starch, the mixture possesses the advantage, in comparison with butanol alone, of moving phenylalanine well ahead of leucine and isoleucine (Fig. 1). With this solvent, however, recoveries of methionine have been low, occasionally by as much as 50 per cent. Oxidation of methionine by traces of peroxides present in the benzyl alcohol appears to be responsible for this effect.¹⁰ Quantitative recoveries of methionine are obtained when butanol is used alone. The recoveries can also be made quantitative in the butanol-benzyl alcohol solvent by the inclusion of 0.5 per cent thiodiglycol (redistilled Kromfax solvent). The solvent mixture which has been adopted for the chromatographic analysis of the first six amino acids (Fig. 1) is made up of 500 cc. of *n*-butanol, 500 cc. of benzyl alcohol, 144 cc. of water, and 5 cc. of thiodiglycol. If the thiodiglycol is not added, the water content is reduced to 142 cc.

At 25°, the above solvent is slightly undersaturated with respect to water. It is essential that all solvents meet this specification. If saturated solvents are used, slight changes in temperature may induce the separation of free droplets of water in the solvent above the chromatogram. The collection of excess water on the top of the starch column can markedly distort the amino acid peaks. The amount of water in the solvent also requires careful control in order to effect optimum separation of tyrosine and valine. If the water content of the above mixture is decreased to 138 cc., the tyrosine and valine peaks are so close together that there is no evidence of a dip or valley in the curve. The curve in Fig. 1 was obtained before this factor was fully appreciated and the result shown is therefore marginal. The increase of the ratio of benzyl alcohol to butanol to 55:45 did not permit a wider variation in the amount of water in the solvent. The relative positions of the other amino acid peaks are not affected by similar small variations in the water content. For experiments with methionine, the solvent should be used within 2 weeks after the addition of the thiodiglycol.

Butanol saturated with water contains about 170 cc. of water per liter. For the chromatogram shown in Fig. 1, *B*, an undersaturated solvent is prepared by diluting 150 cc. of water to 1 liter with *n*-butanol. The acidic solvent for Fig. 1, *C*, was prepared from 170 cc. of 0.57 *N* HCl made up to 1 liter with butanol. Since the presence of HCl increases the solubility of

^a The organic solvents employed in this work have been of analytical reagent grade. Distillation prior to use has been found unnecessary.

¹⁰ The peroxide content of different lots of benzyl alcohol varies as judged by the KI test. Samples giving a strongly positive reaction are not used. Even when the KI test is essentially negative, thiodiglycol must be included in the solvent.

water in the alcohol, this solvent is still slightly undersaturated. The curve with the butanol-HCl mixture has been included in Fig. 1 for comparison. This solvent is unsatisfactory for many purposes, since the recoveries of amino acids may run somewhat low because of esterification.

The solvents described above are only three examples from the variety of mixtures that can be tried with starch columns, depending upon the objectives of the experiment. Further work will be reported on the use of solvents of higher water content for the fractionation of the slower moving amino acids such as alanine and glycine, and the acidic and basic amino acids. These components are still on the column at the end of the experiments described in the present communication.

DISCUSSION

Variations in Starch—The potato starch manufactured by Morningstar Nicol, Inc., which was used in the present experiments, contained 0.3 per cent ash (as sulfate), 0.05 per cent nitrogen, and 12 to 20 per cent moisture. The granules varied in size from 0.01 mm. to 0.06 mm. with the diameter of the average particle being about 0.03 mm. It has been noted that the starch is capable of neutralizing small amounts of HCl. When 20 gm. were suspended in 50 cc. of 0.01 N HCl and an aliquot of the supernatant was titrated, it was found that 1.5 cc. of 0.01 N HCl were neutralized per gm. of starch. Small quantities of ash and other impurities are extracted from the starch by the butanol and butanol-benzyl alcohol solvents. This material does not affect the analytical experiments with starch chromatograms. For isolation work, it may prove desirable to wash the starch four to six times with 6 volumes of distilled water. Before it is dried, the water-washed starch should be washed with ethanol in order to facilitate the production of a finely divided air-dried powder. For analytical experiments, the starch is used as it comes from the manufacturer, as further treatment has not served to improve its resolving power. Samples of starch washed and dried in the laboratory have actually not given quite as good column performance as the untreated commercial material.

Experiments have also been carried out with a sample of potato starch purchased from Messrs. Gordon Slater, Ltd., Manchester, England, which presumably corresponds to the material employed by Synge (6). The ash content was less than 0.1 per cent and the nitrogen content was 0.05 per cent. The particle size was significantly larger than that noted above. The average granule had a diameter of about 0.04 mm. When exposed to a saturated atmosphere of water or butanol containing 15 per cent water in a desiccator at 25°, both the Morningstar Nicol and the Gordon Slater preparations adsorbed 50 per cent of their dry weight of water or water plus

a small amount of butanol. This value is higher than the value of 35 per cent reported by Synge (6).

In chromatographic work, the reproducibility of the adsorbent is a key factor in determining the usefulness of a given fractionation procedure. In the present experiments with starch, it has been found that almost identical results can be obtained with different batches of potato starch from different manufacturers when measures are taken to compensate for variations in trace metal contamination and in particle size.

The effect of small amounts of amino acid complexes of metal ions was observed in early experiments in which the first peak through the column was markedly distorted (10). It was found that this difficulty could be completely overcome by removing the interfering ions with 8-hydroxyquinoline. When a chromatogram was run with the Gordon Slater starch without treating it with hydroxyquinoline, the effect of the metal ions was not limited to the first peak, but rendered the whole chromatogram completely unsatisfactory. In butanol-benzyl alcohol the first three components emerged as one broad zone with a recovery of only 55 per cent. Prior hydroxyquinoline treatment yielded a curve almost indistinguishable from that in Fig. 1. The resolution was nearly as good and the peaks were in the same positions. With this starch, however, it was necessary to use 4 times as much hydroxyquinoline as that prescribed for the Morningstar Nicol starch. Therefore, if poor resolution is obtained with a given starch, an increase in the quantity of hydroxyquinoline may be tried. If possible, it is preferable to procure a sample of starch which requires only the minimum treatment with hydroxyquinoline. The metal effect is not correlated with the total ash of the starch. The interference is due to a minor inorganic component and it is not necessary to carry the hydroxyquinoline extraction to the point of completion. To continue the extraction to the point where no more colored complexes are eluted may require about 10 times the amount of hydroxyquinoline necessary to yield optimum amino acid resolution.

Columns packed with the Gordon Slater sample of starch flowed at about twice the optimum rate when poured originally with 30 per cent water content. The increase in flow rate correlates with the observation on the larger granule size. The satisfactory results cited above were obtained on columns poured with an initial 20 per cent water content to compensate for this difference.

Starches other than potato starch have been studied. Commercial corn-starch, after purification by acid-alcohol extraction to remove nitrogenous impurities and lipides, gave effluent curves which were qualitatively the same as those obtained with potato starch. The resolving power was much inferior and it was not determined whether improvements in the packing

procedure could be made. Rice starch, with its much smaller particle size, gave flow rates that were inconveniently slow. Canna starch,¹¹ which possesses the largest granule size among the common starches, gave less uniform column flow when tested with a colored sample zone. On the basis of purity, availability, and functional tests, potato starch appears to be preferable to other starches for chromatographic work with amino acids.

Techniques of Column Packing—The degree of resolution of phenylalanine, leucine, and isoleucine in the butanol-benzyl alcohol solvent mixture has been used as a test in studies on the effect of variations in the packing procedure. With a poor column, the peaks are in the same positions but are lower and the spreading of the zones tends to fill in the valleys. The use of electric vibrators during packing, rotation of the column during pouring, or continuous agitation of the slurry during its introduction appeared to have little influence on the results. After the accumulation of observations on a large series of columns of varying efficiencies, the most important point in the packing procedure proved to be the water content of the starch at the time the column was prepared. Control of the water content affects the uniformity of the packing in two ways. First, the density of the starch granule decreases with increasing hydration. The density of the particles at 20 to 30 per cent water content is such that they settle very slowly and evenly in dry butanol, but the density difference is not so small that the granules tend to "float" in the solvent. The observation of a sharp settling line moving up the chromatograph tube is evidence for a satisfactory density difference. Secondly, the swelling of the starch granules from about 30 per cent water content to 50 per cent, which occurs after the column has settled to constant height and while it is equilibrating with the wet organic solvent, creates an evenly distributed internal pressure capable of reducing the intergranule interstices. The high efficiencies of these columns result in large part from the particular properties of the starch granule which make possible a packing procedure of the type employed.

As the initial water content of the starch during the pouring of the column is decreased below 20 to 30 per cent, no further significant increase in column efficiency has been obtained. The packing procedure outlined in the experimental section routinely gives columns which are essentially identical in flow characteristics and resolving power. This generalization holds for columns up to 8 cm. in diameter, the characteristics of which can be accurately predicted from runs on columns 0.9 cm. in diameter.

Rate of Flow—Decrease of the rate of flow below that prescribed in the experimental section has not yielded improved resolution. Doubling the

¹¹ We are indebted to Mr. C. V. Caesar of the Stein, Hall and Company, Inc., New York, and to Dr. C. O. Beckmann for the samples of canna starch.

flow rate by increasing the pressure has caused about a 10 per cent decrease in the heights of the peaks and corresponding spreading into the valleys. This increased flow rate requires the use of a piston assembly of the type described by Claesson (12) to avoid contact between the solvent and the air at the higher pressure. The solvent was added from a 100 cc. glass syringe attached to the top of the column. The syringe was enclosed in a brass container fitted to the upper part of the chromatograph tube through a rubber stopper. The maximum pressure of 15 cm., which has been adopted for the present experiments, is convenient from the standpoint of operation and gives optimum resolution.

Variations in Amino Acid Sample—Successful fractionation of a mixture of amino acids on a starch column depends upon not overloading the column. The quantity of material that can be handled will vary with the composition of the mixture and the objective of the experiment. With the protein hydrolysates studied in the present experiments, the maximum total load of 2.5 mg. on a butanol-benzyl alcohol column causes a narrow white zone to form at the top of the translucent column. This zone is associated with the liberation of small amounts of water which cannot be absorbed by the slightly undersaturated solvent. If the amino acid load is doubled, the amount of water liberated may be sufficient to cause visible streaks to spread down the walls of the tube. Under these conditions, the peaks emerging in the effluent are broadened and resolution is inferior. The glycine, glutamic acid, lysine, ammonium chloride, and other very hydrophilic components of the mixture contribute to the limiting load in this case. The presence of significant percentages of NaCl, or other inorganic salts, may have the same effect.

The loading of phenylalanine, leucine, and isoleucine can be increased in the present experiments, if these constituents are the only components of the mixture. If the objective of the experiment is the determination of methionine, which gives a peak well separated from the rest, the load can be increased without regard to some reduction in resolving power. Also, if the objective is the isolation of components from the effluent of a large column, columns can be operated at a higher capacity and the overlapping zones discarded in working up the fractions.

Identification of Amino Acid Peaks—The effluent volume at which a given amino acid peak emerges from the column has been established in a series of experiments with simple and complex known mixtures. In experiments with relatively simple unknown mixtures, such as acid hydrolysates of purified proteins, the considerations described below make it possible to identify the familiar amino acid peaks with a relatively high degree of certainty. It should be stated at the outset, however, that in chromatographic work with unknown mixtures the only unequivocal method for the

qualitative identification of the component or components in a peak is through isolation of the material in sufficient quantity to permit its characterization by independent microchemical methods. The fact that the starch column can be scaled up to 8 cm. or more in diameter indicates that this approach is feasible. Further work is required on techniques for the isolation of components from the effluent on a preparative scale.

Identification of the peaks is facilitated by the fact that the effluent volume for a given amino acid has been found to be constant to within ± 5 per cent, depending upon the nature of the solvent and the weight of starch used in the preparation of the chromatogram. The position of an individual amino acid has not been influenced by the presence of other amino acid components in the mixture being fractionated. Leucine, for example, emerges at the same position when added in a synthetic mixture as when added alone. In the graphical presentation of the results, it has been convenient to measure the effluent volume from the time the sample is placed upon the column, without subtracting the initial column volume. Small variations in the positions of the effluent peaks are thereby introduced as a result of differences in the tightness of the column packing. The variations from this source are negligible in the present experiments.

Comparison of the knowns and unknowns with respect to the general sequence of the peaks and their precise positions relative to one another is of importance (Figs. 1, 3, and 4). The position of the leucine peak relative to the neighboring phenylalanine and isoleucine peaks is even more reproducible than the absolute effluent volume. The exact position of a peak on the abscissa may change slightly, for example, with small variations in the composition of the solvent. The relative positions of two peaks are seldom similarly sensitive. An exception is the case of tyrosine and valine discussed earlier.

The positions of the peaks obtained in the analysis of an unknown mixture can be checked by the addition of one or more known amino acids to the sample prior to analysis. The corresponding peaks on the effluent curve should rise without loss of symmetry, and the added amount of amino acid should be recovered quantitatively. In some cases, information on the identity of a peak can be obtained by specific color reactions. In the tyrosine range, where frequently only every second fraction is used for ninhydrin analysis, the remaining fractions containing tyrosine can be pooled, concentrated to dryness, and tested with Millon's reagent. The probability of correct identification can also be greatly increased by demonstrating that the peak from the unknown appears at the correct position when chromatographed with two or more different solvents.

When working with mixtures more complex than the usual protein hydrolysate, the problems of identification are increased. Conclusions

based upon chromatographic data alone should be made with caution, particularly in the case when the mixture has not been subjected to hydrolysis. In such instances, in the absence of additional information, it cannot be assumed that a peak in the effluent curve occurring in the phenylalanine range, for example, is phenylalanine. Nevertheless, information useful as a basis for further work may be obtained. Care in the examination of each peak for its absolute position, its position relative to other peaks, and any unusual degree of asymmetry may reveal the presence of unsuspected components. If a peak should occur in a portion of the curve normally unoccupied by any of the common amino acids, it is possible to state definitely that it is not one of these substances. Similarly, the absence of an amino acid can be unequivocally demonstrated within the accuracy of the ninhydrin method. It must be borne in mind that the ninhydrin method of analysis is sensitive only to compounds containing amino groups. A single symmetrical peak on the effluent curve does not exclude the presence of ninhydrin-negative compounds with similar rates of travel on the chromatogram.

Among the possible fast moving components other than those shown in Fig. 1 is tryptophan which emerges near to phenylalanine in the three solvent mixtures. In the butanol-benzyl alcohol solvent, this amino acid is readily detected in the valley between phenylalanine and leucine, and in Fig. 4, *A* would yield a peak at an effluent volume of 38.5 cc. If the separation of phenylalanine, tryptophan, and leucine is required, experiments have indicated that they can be completely differentiated by rechromatographing the mixture on an aqueous 0.1 *N* HCl column (10). With acid hydrolysates of proteins, the problem seldom arises, since tryptophan is usually decomposed during the hydrolytic process. Other possible amino acid components in the leucine-valine range include dibromo- and diiodo-tyrosine. The latter emerges at an effluent volume of 16 cc. (Fig. 4, *A*). The presence of peptides, of course, would introduce many possible additional components.

There are no detectable differences in the rates of travel of D-, L-, and DL-amino acids on the starch column. This point has been checked with the L and DL forms of all of the amino acids covered by the present experiments, except tyrosine.

The positions of the amino acid peaks are also of significance in the theoretical interpretation of the action of the starch column. It has been pointed out that the rates of travel of the amino acids on the column do not correspond in all cases to the rates to be expected from the liquid-liquid distribution theory (10). Further studies on the acidic and basic amino acids have revealed similar discrepancies. The available data suggest that the underlying principles governing the operation of the starch column are

adequately covered by the currently accepted definition of the chromatographic process. The term "liquid-liquid (partition) chromatography" does not appear to be applicable to the starch column.

Quantitative Analysis of Synthetic Mixtures of Amino Acids—The results obtained in a series of chromatograms performed on synthetic mixtures containing the eighteen amino acids most commonly found in protein hydrolysates are summarized in Table I. Recoveries on columns 1.9 cm. and 0.9 cm. in diameter have been included. The results indicate that in work with protein hydrolysates, an individual determination of a component present to the extent of 3 per cent or more of the protein is seldom in error

TABLE I

Recoveries of Amino Acids from Mixtures Containing Nineteen Components
Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water.

Mixture	Per cent recovery					
	Phenylalanine	Leucine	Isoleucine	Methionine	Tyrosine	Valine
Synthetic β -lactoglobulin hydrolysate*	101.4	101.0	103.5		97.9	102.0
	97.3	100.4	104.3		99.0	99.8
	98.6	104.5	106.8		101.8	103.0
Synthetic bovine serum albumin hydrolysate†	103.5	102.8	100.2		105.3	98.3
	103.6	101.0	102.0		106.4	96.7
	101.8	101.6	103.6		100.6	99.0
	98.4	102.0	100.0	100.0‡	101.1	100.1
	99.4	101.0	102.8	100.6‡	102.7	100.8
Average.....	100.5	101.7	102.9	100.3	101.8	100.0

* Corresponding in composition to an acid hydrolysate of β -lactoglobulin (Brand *et al.* (7)). Tryptophan was omitted.

† Corresponding in composition to an acid hydrolysate of bovine serum albumin (Brand (13)). Tryptophan was omitted.

‡ The solvent contained thiodiglycol. The amount of methionine present was increased to 6 times that reported for bovine serum albumin.

by as much as 5 per cent. The averages of several determinations have given an accuracy of ± 3 per cent.

Composition of Hydrolysates of β -Lactoglobulin—The sample of β -lactoglobulin used in these experiments was prepared in the laboratory of the late Dr. Max Bergmann by Dr. G. Haugaard and was one of two samples recently analyzed by Brand and coworkers (7). The figure of Brand *et al.* for the nitrogen content of the ash- and moisture-free protein, 15.6 per cent, was confirmed and was employed as a basis for calculations in the present work. This protein offered opportunity for comparison of the analytical results obtained by chromatography on starch with those obtained by other methods.

The effluent curve from a sample of the hydrolysate showed no unexpected peaks (Fig. 3). The positions of the six peaks present corresponded

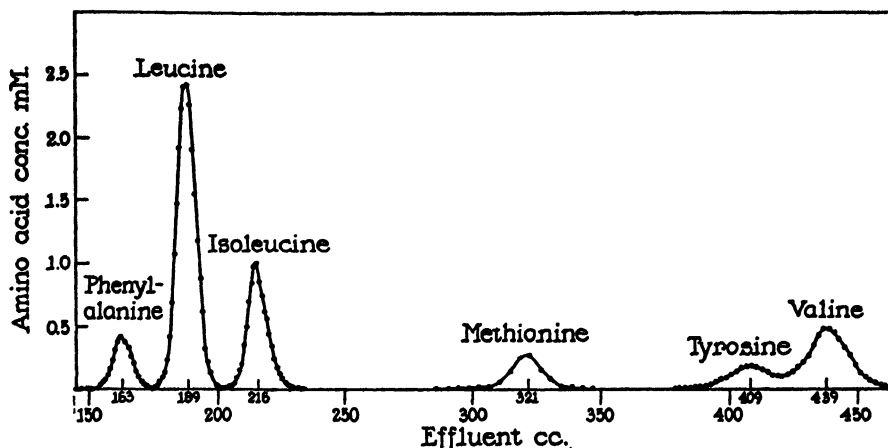


FIG. 3. Chromatographic analysis of a hydrolysate of β -lactoglobulin. Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water (without thiodiglycol). Column, 52 gm. of starch (anhydrous); diameter, 1.9 cm.; height, 30 cm. Sample, about 20 mg. of amino acids.

TABLE II

Amino Acid Composition of Hydrolysates of β -Lactoglobulin

The numbers in parentheses refer to the bibliography.

Method of determination	Hydrolysate No.	Amino acid, gm. per 100 gm. protein				
		Phenylalanine	Leucine	Isoleucine	Tyrosine	Valine
Chromatography on starch	1	3.74	15.4	5.81	3.58	5.71
	2	3.82	15.7	6.04	3.81	5.61
	2	3.77	15.5	5.74	3.52	5.55
Average.....		3.78	15.5	5.86	3.64	5.62
Isotope dilution			15.7(14)			
Microbiological		3.54(7)	15.4(15)	8.4(7)		5.8(7)
		4.3(16)	15.3(16)	7.0(16)		5.5(16)
				8.7(17)		
				6.1(18)		
Chromatography on silica gel		4.2(3)				5.8(3)
Solubility product			15.9(19)			
Photometric					3.78(7)	

to those of recognized components of the protein. When known amounts of phenylalanine and isoleucine were added to the hydrolysate, the peaks

assigned to these components rose accordingly to give recoveries of 99 and 98 per cent, respectively, for the added quantities. The curve was comparable to that obtained with the synthetic control (Fig. 1, A). The quantitative values obtained by integration of the curves are given in Table II.

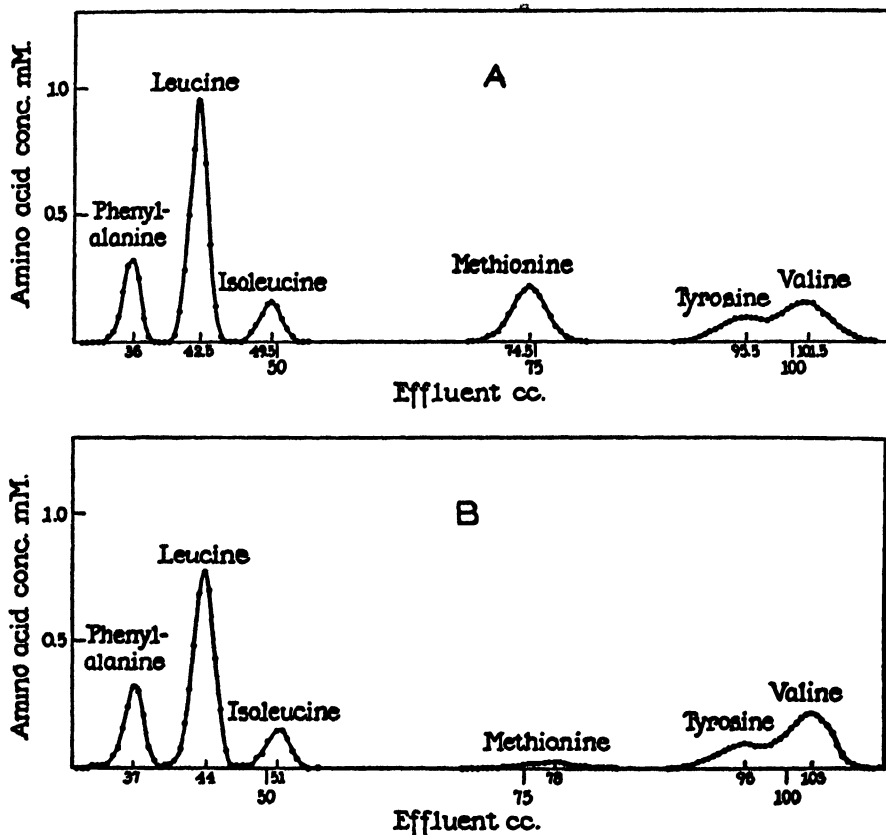


Fig. 4. Chromatographic analyses of a hydrolysate of bovine serum albumin and a synthetic mixture of similar composition. Solvent, 1:1:0.288 *n*-butanol-benzyl alcohol-water, containing 0.5 per cent thiodiglycol. A, synthetic mixture corresponding to an acid hydrolysate of bovine serum albumin with increased methionine content. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, 31 cm. Sample, about 2.5 mg. of synthetic mixture. B, hydrolysate of bovine serum albumin. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, 33 cm. Sample, corresponding to about 2.5 mg. of protein.

The results are in reasonable agreement with the values obtained by other methods, except in the case of isoleucine for which the chromatographic value was more than 20 per cent below the earlier microbiological values. The reason for the high results (7, 16, 17) has subsequently been ascertained

and the most recent value of 6.1 per cent obtained by Smith and Greene (18) is close to the chromatographic figure of 5.86 per cent. Since the β -lactoglobulin experiments were run before thiodiglycol was incorporated into the solvent, quantitative methionine values were not obtained.

Composition of Hydrolysates of Bovine Serum Albumin—The sample of protein analyzed was obtained through the kind cooperation of Dr. Erwin Brand, and was the same preparation (Armour, lot No. 18) which was analyzed in his laboratory. The nitrogen content of the ash- and moisture-free protein, 16.07 per cent, given by Brand (13), was confirmed. An

TABLE III
Amino Acid Composition of Hydrolysates of Bovine Serum Albumin

Method of determination	Hydrolysate No.	Amino acid, gm. per 100 gm. protein					
		Phenylalanine	Leucine	Isoleucine	Methionine	Tyrosine	Valine
Chromatography on starch	1	6.96	12.4	2.65		5.15	6.04
	1	6.42	12.4	2.67		4.76	5.86
	1	6.56	12.6	2.41		4.85	5.92
	2	6.39	11.9	2.52		4.96	5.70
	2					5.30	5.69
	3	6.56	12.0	2.74	0.92	5.18	5.95
	3	6.72	12.3	2.68	0.92	5.24	6.27
Average.....		6.60	12.3	2.61	0.92	5.06	5.92
Other methods		6.2*	13.7*	2.9*	0.81†	5.49‡ 5.53§	6.5*

* Microbiological assay (13, 20).

† Iodometric determination (13, 20).

‡ Photometric determination (13, 20).

§ Isotope dilution method (21).

effluent curve on an acid hydrolysate of bovine serum albumin is given in Fig. 4. Integration of the peaks yielded the results given in Table III. In this case the chromatographic values for leucine, isoleucine, tyrosine, and valine are all about 10 per cent below the values given by Brand (13, 20). Phenylalanine runs about 6 per cent higher and the methionine value is essentially a check. However, the methionine peak was far too low for accurate integration. With a peak as low as that of methionine in this case, an error of 0.005 in the optical density reading for the base-line of the peak could cause an error of 15 per cent in the recovery. For amino acids present in small amounts, the accuracy can be increased by placing a larger sample on the column. For tyrosine, the value of Brand and coworkers

(20) checks well the figure of Shemin (21) who employed the isotope dilution method. In the latter case, however, a different lot of serum albumin was employed. The uniformity of the discrepancy between some of the earlier results and those reported here stimulated a diligent search for systematic errors in the chromatographic procedure. Three different hydrolysates were analyzed with concordant results. The nitrogen content of the hydrolysates was determined by the same micro-Kjeldahl procedure used in the analysis of the protein. The synthetic mixtures gave excellent recoveries (Table I). There appears to be no systematic explanation for the differences.

SUMMARY

A procedure for the quantitative chromatographic separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine has been developed. The amino acid mixture is fractionated on a column packed with potato starch. The solvent which has been used in most of the experiments is 1:1:0.288 *n*-butanol-benzyl alcohol-water containing about 0.5 per cent thiodiglycol.

Photometric ninhydrin analyses are performed on small increments of the effluent solution to permit the construction of effluent concentration curves which reveal in detail the changes in composition of the eluate. With columns 0.9 cm. in diameter and 30 cm. in height, integration of the resulting peaks gives amino acid recoveries accurate to 3 to 5 per cent in individual determinations on 0.1 mg. quantities of a component. The average of several experiments gives recoveries to within ± 3 per cent on synthetic mixtures of nineteen components corresponding in composition to protein hydrolysates. Experiments have been carried out on the determination of the amino acid composition of acid hydrolysates of samples of bovine serum albumin and β -lactoglobulin.

An automatic fraction-collecting machine is described for the collection of the large number of small effluent fractions required in this type of chromatography. The techniques for measurement of the shape and position of the emerging peaks have permitted careful comparison of different samples of starch and the factors which enter into the preparation of uniform columns. It has been possible to define procedures which have given fully reproducible resolving power from column to column and with different preparations of potato starch. The columns can be scaled up to 8 cm. in diameter without loss of efficiency.

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PHOTOMETRIC NINHYDRIN METHOD FOR USE IN THE CHROMATOGRAPHY OF AMINO ACIDS

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For the investigations on the chromatographic separation of amino acids outlined in the preceding communication (1), it was necessary to have available a suitable quantitative method for the determination of the concentration of amino acids in the effluent from the column. For this purpose, the method should be sufficiently general to include the determination of most of the amino acids and peptides likely to be encountered in protein hydrolysates or other material of biological origin. The method should have as high a sensitivity as possible to permit the determination of low concentrations of amino acids in the effluent from the chromatogram. Also the laboratory procedure should be fairly simple to permit the method to be applied conveniently to large numbers of effluent samples.

It appeared probable that a photometric method would best fulfil these requirements. The two colorimetric methods of this type which had received the most study were the procedures based on the use of β -naphthoquinonesulfonic acid and ninhydrin (triketohydrindene hydrate) as reagents. For reasons which will be described, the ninhydrin reaction was selected for further investigation.

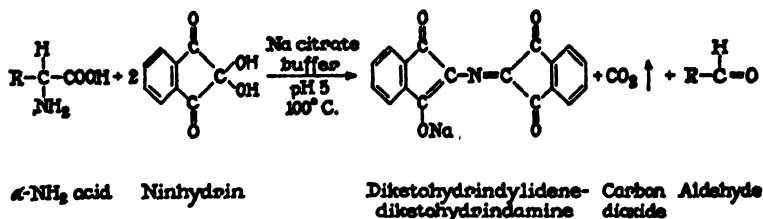
The color reaction between α -NH₂ acids and ninhydrin has been studied extensively in the past. It has been established that colored compounds are formed not only with amino acids, but also with peptides, proteins, and other classes of substances possessing free amino groups. The reaction is known to be extremely sensitive for qualitative work. In earlier attempts to render the color reaction quantitative (2-8), however, it has been found that the color yield per microgram of amino acid decreased markedly as the concentration of amino acid was reduced. In addition, the results have not been reproducible. In the present investigations, it has been observed that, when the color development is carried out in tubes exposed to the air, these difficulties appear to result primarily from the influence of dissolved oxygen. Improved results can be obtained when the reaction is performed in tubes evacuated to 20 mm. Under these conditions, the relationship between color yield and amino acid concentration is more nearly linear, although the deviations are still marked. By the addition of a strong reducing agent directly to the reaction medium, however, the oxidative side reaction has been eliminated. In preliminary experiments

the reduced form of ninhydrin, as hydrindantin (9), was added. Subsequently, it proved simpler to add stannous chloride to the reaction mixture. The stannous chloride serves to reduce part of the ninhydrin, and the preparation of hydrindantin in crystalline form is not required. Although ascorbic acid used as an antioxidant increases the sensitivity of the reaction for qualitative use (10), it is unsuitable for quantitative work because it can also give colored condensation products with amino acids (*cf.* (11)).

When conditions had been established which would give thoroughly reproducible photometric readings with a given amino acid, a study was made with several amino acids to determine the effects upon the reaction of variations in pH, temperature, time of heating, and amounts of reagents. At the same time, the mechanics of the procedure were developed to permit the analysis of large numbers of samples in a routine manner.

The method still possesses one important disadvantage. Although reproducible results are obtained for a given amino acid, the different amino acids do not all yield the same amount of color per mole. For chromatographic analysis, this is not a serious disadvantage. In those instances in which the starch column can separate an amino acid completely from the other components of the mixture, the ninhydrin reaction can be made to give quantitative values by the use of a factor appropriate for the amino acid in question.

Some of the possible causes for variations in the color yield per mole have been studied. The absorption spectra indicate that all the α -NH₂ acids (except cysteine) give the same major colored end-product. This blue coloring matter was prepared by Ruhemann (9) by the reaction of ninhydrin with alanine and by the condensation of hydrindantin with ammonia. To this compound Ruhemann assigned the structure of diketohydrindylidene-diketohydrindamine. In the present work, the product has also been isolated from the mixtures obtained in the reactions of ninhydrin with glycine and glycylleucine. The substance crystallizes as the sodium salt from the



citrate buffer solution used in the present experiments. The absorption spectrum of a standard solution of diketohydrindylidene-diketohydrindamine can be compared with the spectrum of the unfractionated reaction mixtures from ninhydrin and α -NH₂ acids. The comparison shows that in the leucine reaction, for example, the colored product is formed in only 93

per cent of the theoretical yield. A modification of the method which would render the yields quantitative in all cases would be an improvement. For a given amino acid, the percentage yield of the colored product is independent of the initial amino acid concentration. This fact indicates that the low yield is characteristic of the mechanism of the reaction under the experimental conditions employed and is not due to destruction of part of the color by a trace of oxygen.

With proline and hydroxyproline, as shown by Grassmann and von Arnim (12), the reaction follows a different course than with the amino acids containing an α -NH₂ group. These two amino acids give products with a maximum absorption at 440 m μ . The present procedure can also be used to determine proline and hydroxyproline, although the sensitivity is less than in the case of the amino acids which form diketohydrindylidene-diketohydrindamine.

It has long been known that colorimetric ninhydrin methods are not specific for the NH₂ groups of amino acids. The NH₂ groups in peptides give good color development, many amines such as histamine and tyramine will react and the presence of hydrindantin, used in this procedure, causes NH₃ to give a nearly quantitative yield of the blue reaction product. For chromatographic experiments with amino acids and peptides, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity would be a disadvantage, as was recognized by Harding and MacLean (2). The specificity of the photometric ninhydrin method is similar in a number of respects to that of the nitrous acid reaction for amino nitrogen. The method may be of value in instances when the nitrous acid reaction is useful. For the estimation of free amino acids in the presence of peptides, the photometric method, of course, lacks the specificity of the gasometric amino acid carboxyl determination of Van Slyke, Dillon, MacFadyen, and Hamilton (13).

The photometric ninhydrin method, with the present modifications which have rendered the results fully reproducible, appears to possess some advantages over β -naphthoquinonesulfonic acid procedures for those applications for which these methods are suitable. The reaction of amino acids with β -naphthoquinonesulfonic acid, as employed by Folin, recently modified by Frame, Russell, and Wilhelmi (14), and compared with the CO₂ method by Chinard and Van Slyke (15), involves the additional operation of bleaching of the excess reagent. The ninhydrin reagent solution possesses the advantage of being stable, and for routine use can be stored under nitrogen for a month or more. Fading of the color in the ninhydrin method proceeds at a much slower rate than that reported for the naphthoquinone procedure (15). The ninhydrin reaction yields the same end-product from all the α -NH₂ acids (cysteine excepted), whereas the chemistry of the β -naphtho-

quinonesulfonic acid reaction is less well defined and the absorption maxima of the colored products obtained from different amino acids, though similar, are not identical (14).

Apparatus

Pipettes—For the pipetting of large numbers of small samples of amino acid solutions for analysis, modified self-adjusting transfer pipettes are used in 0.05, 0.1, 0.2, and 0.5 cc. sizes.¹ The accuracy of these pipettes is increased by operating them on a manifold connected to both compressed air and vacuum lines. For long series of analyses, this arrangement is also much more convenient for the operator. The arrangement of the pipetting stand is illustrated in Fig. 1. The manifold is made from three T-tubes. The third tube is mounted behind the rubber stopper (size 13). The connections are made with soft rubber hemocytometer pipette tubing. A slight vacuum (or pressure) is applied to the micro pipette by touching the top of the appropriate T-tube and greater vacuum (or pressure) by simultaneously pressing on the adjacent rubber tubing. The stop-cocks are closed only during the adjustment of the vacuum to about 60 mm. and the pressure to about 15 mm. Before use, the tip of the micro pipette should be bent, as in Fig. 1, and fire-polished to give a delivery time of 8 to 12 seconds at 15 mm. pressure. At this rate, and with wiping of the pipette tip before delivery, the reproducibility of delivery is 0.1 to 0.2 per cent. The hold up is about 2 per cent. A series of tubes from a chromatogram is run through without washing the pipette between samples. For each solvent employed the pipette must be calibrated gravimetrically. A table of calibration factors expressed in terms of the fraction of the rated delivery is prepared for each pipette. The metal holders for the 150 × 18 mm. sample tubes and photometer tubes are cut from brass tubing of 20 mm. inner diameter.

Photometer Tubes—For the chromatographic procedure, it has been necessary to accumulate a matched set of over 1000 tubes for use with the Coleman junior spectrophotometer, model 6-A. The tubes have been selected from strain-tested soft glass test-tubes, 150 × 18 mm., without lips.² A solution of methyl red in 0.03 N HCl is prepared of such a strength as to give a reading of 0.60 to 0.70 on the optical density scale when read at 525 m μ against a water zero. About 100 tubes are filled with 5 to 10 cc. of the methyl red solution. It is important that all tubes receive the methyl red solution from the same reservoir bottle. Pouring the solution

¹ The pipettes are made to the design of Dr. P. L. Kirk by the Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California (catalogue No. 283-B).

² Catalogue No. 9446, Arthur H. Thomas Company, Philadelphia, Pennsylvania.

from tube to tube can introduce significant errors. The largest group of tubes giving readings within 0.005 unit of the same value is selected. The side of the tube facing the light source is marked temporarily at the time of the test, and subsequently with a glass-marking tool,³ to indicate the correct position for the tube in the spectrophotometer. About a dozen of

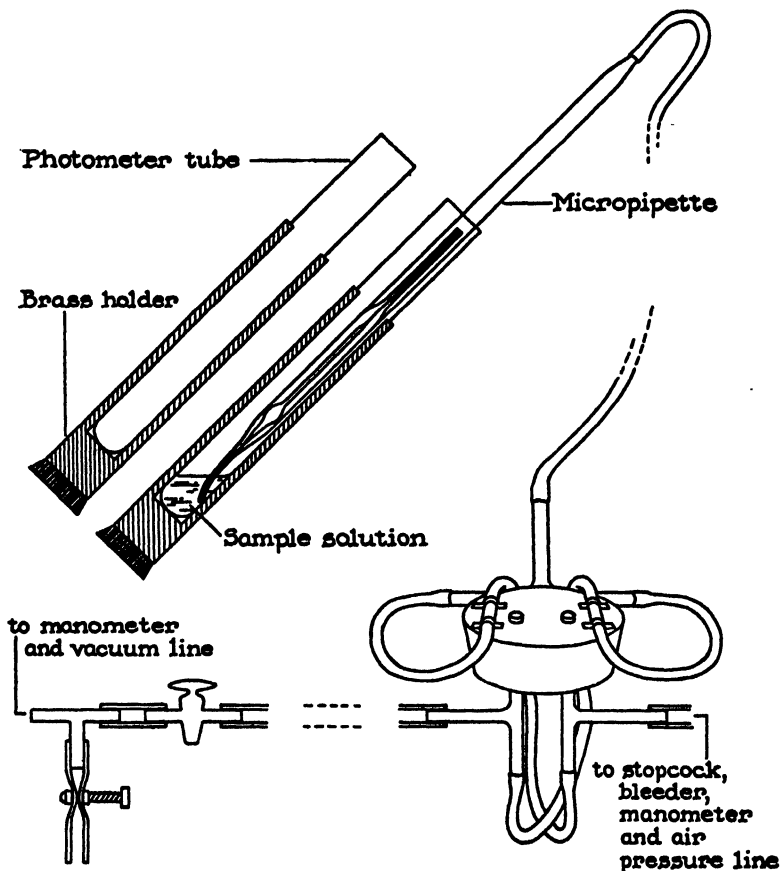


FIG. 1. Arrangement for the pipetting of samples for analysis

these tubes are marked as standards and reserved for use only in the checking of new sets.

In calibrating subsequent groups of 100 tubes against standard tubes filled with methyl red solution, each new tube is rotated in the instrument to determine whether it can be set to read within 0.005 unit of the standard

³ Catalogue No. 3008, Chicago Wheel and Manufacturing Company, 1101 West Monroe Street, Chicago, Illinois, with additional grinding wheels No. 5-B-44.

reading. The satisfactory tubes are appropriately marked for position. The selected tubes are permanently numbered in sets of 200 tubes each. Each set can be subjected to an additional test by observing the zero reading when the tubes are filled with distilled water. In general, tubes selected by the methyl red procedure have given uniform zero readings.

When measured by calipers below the rim, the tubes selected for this study had an inner diameter of 16.25 ± 0.15 mm. The outer diameter averaged 18.3 mm. Once a calibrated set has been selected with tubes of a given average diameter, such as 18.3 mm., it is necessary that subsequent lots of tubes obtained from the manufacturer have an average diameter in the same range. Under these conditions, twenty-five to 50 tubes per hundred may prove acceptable. If such specification is not made, some lots may run all high or all low.

When the sets have been handled in wooden or aluminum racks,⁴ in order to protect the tubes from being scratched, and washed with boiling soap solution, the tubes have remained accurate during constant use for more than a year.

To cover the photometer tubes during the analysis, Aloe-Willett aluminum caps⁵ are used.

Water Bath—A vigorously boiling water bath is required. An open bath can be used, but the amount of evaporation of the solvent (cf. Table III) from the tubes is less reproducible. The present experiments have been carried out in a specially designed, electrically heated, covered bath⁶ with a constant level regulator. For these analyses, the bath is operated with the thermostat at the maximum setting, so that heat is constantly, not intermittently, applied. The tubes are immersed to a depth of about 2 inches. The rate of heat supply should be sufficient to bring the bath back up to 99–100° within 2 minutes after the insertion of a full rack of 50 tubes. Only one rack should be inserted at a time. The rear corners of the cover are bent downward slightly to allow the escape of steam without the collection of condensed water. A double hook handle for insertion and removal of the racks can be made from a $\frac{1}{4}$ inch metal rod.

Pipetting Machines for Reagents—For a small number of analyses, the reagents can be added by a burette or pipette. When large numbers of samples are being run, the ninhydrin solution can be stored under nitrogen in a 1 liter brown or red glass reservoir attached to a pipetting machine.⁷

⁴ Suitable aluminum racks are described in the preceding communication (1).

⁵ Catalogue No. JL-78300 (for 18 mm. tubes). A. S. Aloe Company, St. Louis, Missouri.

⁶ Catalogue No. 405/3/R, Electric Heat Control Apparatus Company, 507 Fifth Avenue, New York 17.

⁷ Model No. 40-SS-10, Brewer Automatic Pipetting Machine, Baltimore Biological Laboratory, Inc., 500 North Calvert Street, Baltimore 2, Maryland.

The flexible connections are made with $\frac{1}{8}$ inch inner diameter Neoprene tubing. A 250 cc. dropping funnel, for use in filling the reservoir, is mounted on the bottle through a 3-hole rubber stopper. The reservoir is connected to the top of a second bottle of the same size by a U-tube, and the second bottle is connected through a 2-hole stopper to a third 1 liter bottle by a U-tube which reaches to the bottom of each. The second bottle is initially filled with water, and the air in the system is replaced by nitrogen introduced through the dropping funnel at the time the apparatus is set up. The reservoir can be refilled without replenishing the nitrogen, except when the apparatus is disassembled for cleaning. The pipetting machine is equipped with a 3 cc. syringe and set for 1 cc. delivery. The valves (ungrooved) may require regrinding by hand with a fine emery or rouge to insure smooth performance with organic solvents. Fire polishing of the glass delivery tip, when small volumes such as 1 cc. are being delivered, helps to eliminate a hanging drop or back flow.

A second pipetting machine, equipped with a 10 cc. syringe, is used to deliver 5 cc. of the diluent solution from a 2 liter storage bottle. The flexible connections can be made with $\frac{3}{32}$ inch inner diameter Tygon tubing or $\frac{1}{8}$ inch Neoprene tubing. For convenience in the analysis of large numbers of samples, the reaction mixtures are diluted with a given volume of solvent from the pipetting machine to avoid the procedure of bringing the solutions to a prescribed volume in calibrated glassware.

Reagents

Ninhydrin—To insure a low blank reading in the photometric procedure, the ninhydrin, prepared commercially according to the method of Teeters and Shriner (16), has usually been recrystallized within several months of the time of use. To 250 cc. of water, 100 gm. of ninhydrin are added. The hot solution is treated with about 5 gm. of decolorizing carbon. The filtrate is stored at 4° overnight. The ninhydrin is washed on the filter four or five times with 20 cc. portions of cold water. The air-dried crystals are stored in dark glass. The recovery is 85 to 90 per cent.

Citrate Buffer—The buffer, pH 5 (0.2 M), is prepared from 21.008 gm. of citric acid, $C_6H_8O_7 \cdot H_2O$ (reagent grade) and 200 cc. of N NaOH diluted to 500 cc. Several times this quantity can be prepared and stored in the cold with thymol. The pH of the buffer when diluted with an equal volume of water should be 5.0 ± 0.1 .

Methyl Cellosolve—The samples of methyl cellosolve should give a clear solution when mixed with an equal volume of water³ and should give a negative or very faint peroxide test with 10 per cent aqueous KI.

³ Turbidity from several samples of methyl cellosolve was found to result from the fact that the solvent had been repackaged in lacquered cans. Purchase of the solvent packaged in glass or in the manufacturer's original containers is to be preferred.

Ninhydrin Solution—Dissolve 0.80 gm. of reagent $\text{SnCl}_4 \cdot 2\text{H}_2\text{O}$ in 500 cc. of the citrate buffer, pH 5. Add this solution to 20 gm. of recrystallized ninhydrin dissolved in 500 cc. of methyl cellosolve. Transfer the reagent solution to the reservoir bottle. If the system is not already filled with nitrogen, run a stream of nitrogen through the dropping funnel for about 30 minutes. When stored in this manner the solution can be kept for at least a month without deterioration. The adequacy of the preservation of hydrindantin in the reagent solution can be checked by determining the color yield on a standard amino acid solution of relatively low concentration (1 mm). If unrecrystallized ninhydrin is used, and the reagent solution is clarified with carbon, high blanks are likely to result from nitrogenous materials on the carbon.

If only a few analyses are being run at one time, and a pipetting machine is not required, the necessary small quantity of the reagent solution can be prepared for immediate use and the 1 or 2 cc. aliquots run into the photometer tubes from a burette.

Diluent Solution—Mix equal volumes of water and *n*-propanol (c.p.).

Standard Amino Acid Solutions—For the analysis of 0.1 cc. samples, 1.6 to 2.0 mm solutions of amino acids are convenient. The solutions are diluted 1:1 or 1:4 for analyses in which 0.2 or 0.5 cc. samples are required. When water-alcohol solvents are employed, the amino acids are dissolved in water and the solutions made to the appropriate volume with the dry organic solvent. A small amount of HCl is added to dissolve tyrosine and cystine. Blank solutions for each solvent mixture are prepared at the same time.

Procedure

In the case of standard solutions of amino acids, triplicate samples of the blank and the standards are pipetted into a series of photometer tubes. When the effluent from a chromatogram is being analyzed, a single sample is pipetted from each fraction. If an automatic fraction collector (1) has delivered the appropriate size of sample directly into the photometer tubes, the pipetting step is eliminated.

If the solutions for analysis are acidic, they should be neutralized (methyl red) to within ± 0.1 cc. of 0.1 *N* NaOH. For a series of tubes from a chromatogram, a preliminary titration is made on a blank sample. Subsequently, 1.0 *N* NaOH is appropriately diluted so that 1 or 2 drops (0.05 to 0.1 cc.) from a burette will bring the sample within the desired limits. The prescribed number of drops of the alkali of adjusted concentration is added to each tube of the series prior to analysis. Acidic solutions will usually pick up a trace of NH_3 from the air. Since as little as 0.1 γ of NH_3 can be detected by the present procedure, it is important that the blanks and the analytical samples be handled under strictly parallel conditions.

The pipetting machine for the ninhydrin reagent is checked for delivery by running five aliquots into a 5 cc. volumetric flask. This is done daily before use of the machine, and also serves to discard the solution which has been standing in the Neoprene tubing. The delivery should be within ± 0.5 per cent of the set value. One or two aliquots of the ninhydrin solution (1 cc. for 0.1 and 0.2 cc. samples; 2 cc. for 0.5 cc. samples) are run into the photometer tubes. With samples in water or in solvents miscible with water, 1 cc. of ninhydrin solution can be used for 0.5 cc. samples if accuracy to the last few per cent is not important. Aluminum caps are placed on the tubes and the tubes are shaken to mix the reagent and sample. If the shaking is done by machine, the rack can be placed for 30 seconds on a reciprocal shaker⁹ operating at about 240 excursions per minute.

The rack of tubes is heated for 20 minutes in a vigorously boiling water bath. The pipetting machine for the water-propanol diluent is checked for delivery with a 25 cc. volumetric flask. 5 cc. (± 0.03 cc.) of the diluent are added to each tube. The stream is directed into the center of the solution to give good mixing. The tubes are wiped and transferred to a dry rack. The tubes are shaken by hand or by machine (1 minute). Readings are taken on the spectrophotometer starting at about 15 minutes after removal from the bath. Readings are essentially constant for 1 hour after completion of the reaction. The analyses should be run in groups of not more than 50 tubes to permit the readings to be completed within this time.

The tubes are read on the Coleman junior spectrophotometer, model 6-A, at 570 $m\mu$. The blanks are read against a reference tube of 1:1 propanol-water. The average blank tube is chosen, and the rest of the series read with the instrument set on the blank determination as zero. The blank reading should be about 0.08 to 0.10 on the optical density scale for 1 cc. of reagent and 0.15 to 0.20 for 2 cc. of reagent. Proline and hydroxyproline are read at 440 $m\mu$.

For tubes which read near to or above 1.00, the solutions and the blank are diluted with additional 5 cc. samples of the propanol-water solution. When the volume in the tube reaches 16 to 17 cc., shaking by machine is not satisfactory, and inversion of the Neoprene-stoppered tubes is required. If the readings are still off the scale (above 1.00), samples are pipetted into other photometer tubes for further dilution.

When an analysis is completed, the tubes are rinsed with water, a monel wire screen or a perforated aluminum cover being used to permit the draining of a full rack of 50 tubes in one operation. The tubes are half filled with 0.2 per cent aqueous solution of soap flakes. The rack of tubes is heated for 20 minutes in a boiling water bath. The tubes are rinsed three times with water and dried in an oven at 110°. The boiling soap solution is

⁹ Catalogue No. 5855, Precision Scientific Company, Chicago, Illinois.

required to remove the band of material that is deposited on the walls of the tubes when volatile solvents are used.

Calculations

A standard curve is plotted for 0.1 cc. aqueous samples of leucine at six concentrations varying from 0.5 to 2.0 mM. Before being plotted, the average values are divided by the pipette calibration factor for water to give corrected readings for 0.100 cc. samples. From the graph, a table is prepared giving the millimolar concentrations corresponding to optical density readings from 0.01 to 1.00, in steps of 0.01 unit. The concentrations are multiplied by 11.1/6.1 and 16.1/6.1 to give concentrations corresponding to the readings obtained after dilution of the 6.1 cc. with one or

TABLE I

Relationship of Optical Density to Leucine Concentration (Condensed Table of Leucine Equivalents)

Determined on 0.100 cc. aqueous samples in photometer tubes of 16.25 mm. inner diameter.

Spectrophotometer reading, optical density × 100	Leucine concentration, mm per liter			Spectrophotometer reading, optical density × 100	Leucine concentration, mm per liter		
	Volume of diluent added				Volume of diluent added		
	5 cc.	10 cc.	15 cc.		5 cc.	10 cc.	15 cc.
10	0.196	0.357	0.518	60	1.18	2.15	3.11
20	0.392	0.714	1.03	70	1.39	2.53	3.67
30	0.588	1.07	1.55	80	1.61	2.93	4.25
40	0.784	1.43	2.07	90	1.83	3.33	4.83
50	0.980	1.78	2.59	100	2.05	3.73	5.42

two additional 5 cc. aliquots of the diluent. A condensed format of the standard table obtained with tubes possessing an inner diameter of 16.25 mm. is given in Table I, which covers concentrations up to 5.4 mM. The procedure can be extended by manual dilutions to much higher concentrations. The curve follows Beer's law through readings up to an optical density of about 0.50. There is a deviation of 4 per cent from the straight line relationship at an optical density of 1.0.

For the other amino acids and related compounds, the color yields per mole can be expressed relative to the leucine value as 1.00. For 0.1 cc. aqueous samples, the millimolar concentrations read from Table I, corrected for pipette delivery, were divided by the millimolar concentrations of the standard solutions to give the yields summarized in Table II. Each amino acid and peptide listed in Table II was checked for correct elementary analysis and, whenever possible, for optical rotation (*cf.* (1)). The other sub-

stances were obtained from commercial sources and were not purified before analysis.

For other than 0.1 cc. aqueous samples, the values given in Table I for millimolar concentration corresponding to a given spectrophotometer reading require correction for the changes in volume involved. Before

TABLE II

Color Yields from Amino Acids and Other Compounds on Molar Basis Relative to Leucine

Determined on 0.1 cc. aqueous samples of 2.0 mM solutions; heating time, 20 minutes; read at 570 $m\mu$.

Compound	Color yield	Compound	Color yield
Alanine	1.01	Glutathione	0.76
Arginine	1.00	Glycine ethyl ester	1.00
Aspartic acid	0.88	Glycyltyrosine	0.88
Citrulline	1.03	Glycylphenylalanine	1.04
Glutamic acid	1.05	Glycylglycine	0.89
Glycine	1.01	Glycylleucine	1.05
Histidine	1.04	Leucylglycine	0.92
Isoleucine	1.00	Phenylalanylglycine	0.97
Leucine	1.00	Phenylalanine ethyl ester	0.98
Lysine	1.12	Histamine	0.65
Methionine	1.00	Taurine	0.97
Phenylalanine	0.88	Tyramine	0.64
Serine	0.94	Sarcosine	0.84 <i>Ca.</i>
Threonine	0.92	Glucosamine	1.00
Tyrosine	0.88	Creatine	0.03
Valine	1.02	Creatinine	0.03
Cysteine	0.15 <i>Ca.</i>	Dibenzylamine	0.04
Half-cystine	0.54	Glycine anhydride	0.01
Tryptophan	0.72 <i>Ca.</i>	Urea	0.03
Proline	0.05	Adenine	0.00
Hydroxyproline	0.03	<i>p</i> -Aminobenzoic acid	0.00
Ammonia	0.98 <i>Ca.</i>	Diethylbarbituric acid	0.00
Asparagine	0.94	Glucose	0.00
Glutamine	0.99	Uric acid	0.00

calculating the correction factors to be applied to the analysis of samples containing volatile organic solvents, it is necessary to determine gravimetrically the amount of evaporation during the analysis by weighing tubes before and after heating the reaction mixture for 20 minutes under the experimental conditions employed in an actual determination. With butanol-water and propanol-water samples, essentially the entire 0.1 to 0.5 cc. sample of solvent evaporates during the heating process. If acidic

samples are neutralized before analysis, the volume of NaOH solution added must be included in the calculation. When each term is expressed in cc., the correction factors (F) are calculated as follows:

$$F = \frac{(\text{Sample volume} + \text{neutralizing solution} + \text{ninhydrin solution} + \text{diluent}) - (\text{loss by evaporation})}{1.1 + \text{diluent}} \times \frac{0.100}{\text{sample volume}}$$

Representative factors for two organic solvents are given in Table III. As a first approximation, these factors, used in conjunction with the rela-

TABLE III
Factors for Different Sample Sizes and Solvent Systems

Factors by which the millimolar concentrations from Table I are to be multiplied to give corrected leucine equivalents.

Solvent	Sample size	Ninhydrin solution	Loss by evaporation		Factor†		
					Volume of diluent added		
					5 cc.	10 cc.	15 cc.
	cc.	cc.	mg.	cc.*			
Water	0.1	1	(14)‡		1.000	1.000	1.000
	0.2	1	(19)		0.508	0.504	0.502
	0.5	2	(19)		0.246	0.225	0.217
Butanol-water§	0.1	1	94	0.10	0.984	0.991	0.993
	0.2	1	194	0.20	0.492	0.495	0.496
	0.5	2	395	0.45	0.231	0.217	0.212
Butanol-benzyl alcohol-water	0.1	1	41	0.03	0.996	0.998	0.998
	0.2	1	62	0.05	0.504	0.503	0.502
	0.5	2	79	0.07	0.244	0.224	0.216

* Approximate.

† To be divided by the calibration factor of the pipette.

‡ The small loss from water samples is subtracted from the loss with organic solvent samples in the calculation of approximate volume change.

§ Butanol-water containing 150 cc. of water per liter ($d^{25}_4 = 0.838$).

|| Butanol-benzyl alcohol-water 1:1:0.288 by volume ($d^{25}_4 = 0.936$).

tive yields of color listed in Table II, give satisfactory results for most of the amino acids in these solvents, if an accuracy greater than ± 5 per cent is not required. The color yields given in Table II and the factors listed in Table III may also be used for approximate results with other solvents that may be tried with starch chromatograms. Under such circumstances, the factors given in Table III for water or the butanol-benzyl alcohol solvent may be used for relatively non-volatile solvents, and the butanol factors for volatile solvents.

However, for accurate quantitative work with those solvents which are

selected as optimum for chromatographic analyses, the exact degree of evaporation must be determined experimentally, and the color yields obtained from a given amino acid must be checked by the user with standard solutions made up in the organic solvent. As may be seen from Table IV, the yields thus obtained may differ by a few per cent from the values obtained with aqueous samples given in Table II. The yields should be determined on the same size of sample being used in the chromatographic experiments, although no differences have been observed between 0.1 cc. and 0.5 cc. samples in the solvents studied thus far. For accurate work with solvents other than water, therefore, the concentration of amino acid given in Table I is multiplied by the appropriate factor from Table III and divided by the appropriate color yield from Table IV.

TABLE IV

Color Yields from Amino Acids in Organic Solvent Solutions on Molar Basis Relative to Leucine in Water

Determined on 0.1 cc. to 0.5 cc. samples; heating time, 20 minutes.

Amino acid	Color yield	
	Butanol-water solvent	Butanol-benzyl alcohol-water solvent
Leucine.....	0.99	1.01
Isoleucine.....	1.00	1.03
Phenylalanine.....	0.85	0.86
Tyrosine.....	0.86	0.87
Methionine.....	1.00	1.01
Valine.....	1.01	1.03

The factors given in Table III and the color yields listed in Table IV are fully reproducible when once determined under given experimental conditions. Except with NH_3 and tryptophan, it is not necessary to run controls with each batch of analyses. If this were not the case, the application to chromatography would be rendered unduly complicated by a need for repeated controls. An occasional check on the recovery of leucine from a known solution serves to confirm the reproducibility of the procedure.

The calculation of the recovery of leucine from a synthetic mixture which has been subjected to chromatographic analysis is given in Table V. Suitable data sheets are mimeographed to facilitate the handling of the results. In this example, the automatic fraction collector has been used with a column 0.9 cm. in diameter to deliver approximately 0.5-cc. samples directly to photometer tubes.

When aliquots are pipetted for analysis from larger effluent fractions, the summations of the uncorrected millimolar concentrations are multi-

TABLE V

Data Sheet, Determination of Leucine

Solvent, 1:1:0.288 butanol-benzyl alcohol-water; ninhydrin solution, 2 cc.; fraction collector, 25 drops = 0.504 cc.; entire fraction analyzed; wave-length, 570 m μ ; readings recorded as optical density $\times 100$.

Fraction No.	Volume of diluent, 5 cc.		Volume of diluent, 10 cc.	Volume of diluent, 15 cc.	Uncorrected amino acid concentration†
	Read against 1:1 water-propanol	Read against blank fraction*			
11	22.0	0	0	0	mm
13	22.2	0			
15	21.1	-1			
35	22.0	0			
36	22.0	0			
37	23.5	1.5			
38	29.0	7.0			
39		24			
40		57			
41		100			
42		140	62	81	0.00
43		150	90		0.03
44		130	102		0.14
45		77	84		0.47
46		28			1.12
47	28.5	6.5			2.22
48	22.0	0			3.33
49	22.5	0.5			4.30
50	22.0	0			3.08
					1.54
					0.55
					0.13
					0.00

Integration: Fraction 43. $4.30 \times 0.216 \times 0.5\ddagger = 0.464$

Sum of Fractions 41, 42, and 44. $8.63 \times 0.224 \times 0.5 = 0.967$

" " " 37-40, and 45-47. $3.98 \times 0.244 \times 0.5 = 0.486$

Total = 1.917 micromoles.

Correction for color yield from leucine in this solvent, $1.917/1.01 = 1.898$ micromoles = 0.249 mg. of leucine.

Leucine added, standard aqueous solution of a mixture of amino acids 40 mm with respect to leucine; 0.495 cc. (0.5 cc. pipette, delivery 99 per cent) diluted to 5.00 cc. and 0.485 cc. (0.5 cc. pipette, delivery 97 per cent) placed on the column in butanol-benzyl alcohol. Theoretical yield = 1.920 micromoles = 0.252 mg.; recovery = 98.8 per cent.

* Fraction 11.

† From the expanded form of Table I.

‡ If 0.5 cc. samples are pipetted, the factor becomes $0.216 \times (\text{volume of effluent fraction})/(\text{pipette calibration factor})$.

plied by the appropriate factors from Table III and by the exact volume of an effluent fraction to give micromoles of amino acid. When the whole

fraction is analyzed, as in this example, the sample volume is equal to the volume of an effluent fraction, and the two terms cancel out. At large effluent volumes, when the peaks cover twenty or more fractions, integrations can be obtained from the analysis of every second fraction.

In order to obtain a graphic picture of the fractionation, the uncorrected millimolar concentrations are used directly for preliminary plotting of effluent concentration curves. The graph gives information on the symmetry of the curves and the degree of fractionation. The curves are plotted before decision is made on the division of the peaks for integration. For publication, the curves in the preceding paper (1) have been replotted, each point being corrected to "leucine equivalents" by means of Tables I and III. When the peaks are completely separated, the curves could also be corrected for color yield, but in the case of incomplete separation of the components, this is not possible. In the preceding paper (1) the method is given for the calculation of the amounts of each component in a series of partially overlapping peaks.

Accuracy—When the calibrations have been carefully made both for the pipettes and the photometer tubes, the readings on 0.2 micromole of an α -NH₂ acid can invariably be reproduced to within 0.02 optical density unit, corresponding to an accuracy of about 2 per cent. In the chromatographic analyses, it is necessary to work, in part, below this optimum concentration range. In a large series of chromatograms, integration of the effluent curves has given recoveries of 100 ± 3 per cent, under favorable conditions, and 100 ± 5 per cent for peaks markedly below the optimum average concentration (1).

Reaction with Proline and Hydroxyproline—The preceding method can be used for the determination of proline and hydroxyproline by measuring the yellowish red products of the reaction at their absorption maximum of 440 m μ . However, the optical density readings are only one-fourth and one-seventh, respectively, of those obtained with equimolar solutions of the α -NH₂ acids. The color development is only 80 to 90 per cent complete in 20 minutes at 100°. Standard curves can be prepared for proline and hydroxyproline with a 30 or 40 minute heating period. In the chromatographic analysis, the tubes are usually heated for only 20 minutes along with the rest of the effluent samples. As a first approximation, millimolar concentrations can be calculated from Tables I and III, just as in the case of readings at 570 m μ , and the values can be converted to proline by multiplying by the factor 3.7 and to hydroxyproline by multiplying by the factor 7.2. The measurement of proline by the ninhydrin reaction is of course possible only when this amino acid is completely separated from the other amino acids, as it frequently is on the starch chromatograms. On the other hand, in view of their low absorption at 570 m μ , small amounts

of proline and hydroxyproline can be present simultaneously with α -NH₂ acids without giving significant interference.

Reaction with NH₃.—In the oxidative deamination of amino acids by ninhydrin, 1 equivalent of the reagent is reduced in the course of the formation of diketohydrindylidene-diketohydrindamine (9). If oxygen could be completely eliminated by evacuation of the system, the maximum color yields would be obtained from amino acids without the addition of any further source of reduced ninhydrin. The yield from NH₃ under these conditions would be low, since, of itself, NH₃ does not give rise to the reduced ninhydrin which is essential for the formation of the colored complex. This accounts for the fact that NH₃ does not react positively in a number of the colorimetric ninhydrin procedures that have been used (4, 5, 7). When hydrindantin exists preformed in the reaction mixture, however, as it does in the present procedure, the color yield from NH₃ is in the same range as that from the amino acids. In contrast to the amino acid reaction, which is independent of the concentration of hydrindantin above a certain minimum level, the color yield from NH₃ increases with the hydrindantin concentration. With the reagent solution used in the present procedure, the color yield from NH₃ reaches about 90 per cent of its maximum value. Since different batches of reagent solution may vary somewhat in hydrindantin content, a control determination on a known NH₄Cl solution must be run simultaneously if it is desired to obtain accurate values on NH₃ solutions by the photometric ninhydrin method.

Experiments on Color Development

Isolation of Diketohydrindylidene-Diketohydrindamine.—The product of the reaction of ninhydrin with glycine at pH 5 was prepared in order to compare its absorption spectrum with that of the unfractionated reaction mixture obtained in the ninhydrin analysis.

The amino acid (75 mg.), dissolved in 10 cc. of water, was heated for 20 minutes at 100° with 700 mg. of ninhydrin dissolved in 20 cc. of citrate buffer, pH 5 (0.2 M). The product (245 mg.) which crystallized from the cooled solution corresponded to 75 per cent of the theoretical yield of the sodium salt of diketohydrindylidene-diketohydrindamine. The same procedure carried out with the peptide leucylglycine (188 mg.) gave the same product in 40 per cent yield. For analysis the sodium salt (50 mg.) was recrystallized from about 15 cc. of hot 1:1 water-*n*-propanol.

C ₁₂ H ₈ O ₄ NNa (325.2).	Calculated.	C 66.5, H 2.5, N 4.3, Na 7.08
Prepared from glycine.	Found.	" 66.2, " 2.6, " 4.4, " 7.07
" " leucylglycine.	"	" 66.6, " 2.6, " 4.3, " 6.94

Absorption Spectra.—The absorption curves have been determined with 0.1 cc. samples of 2 mm aqueous amino acid solutions. The final vol-

ume of the reaction mixture was 6.10 cc. in photometer tubes of 16.25 mm. inner diameter. In Fig. 2 the curves for leucine, serine, and ammonia are compared with the absorption spectrum obtained from an equimolar solution of the crystalline sodium salt of diketohydrindylidene-diketohydrindamine. The solution was prepared by dissolving 1.065 mg. of the sodium salt in 100 cc. of a mixture of the ninhydrin solution and the propanol-water diluent in the proportions of 1:10 and was read against this solvent as the blank. The absorption spectra support the conclusion that the α -NH₂ acids and ammonia yield diketohydrindylidene-diketohydrindamine

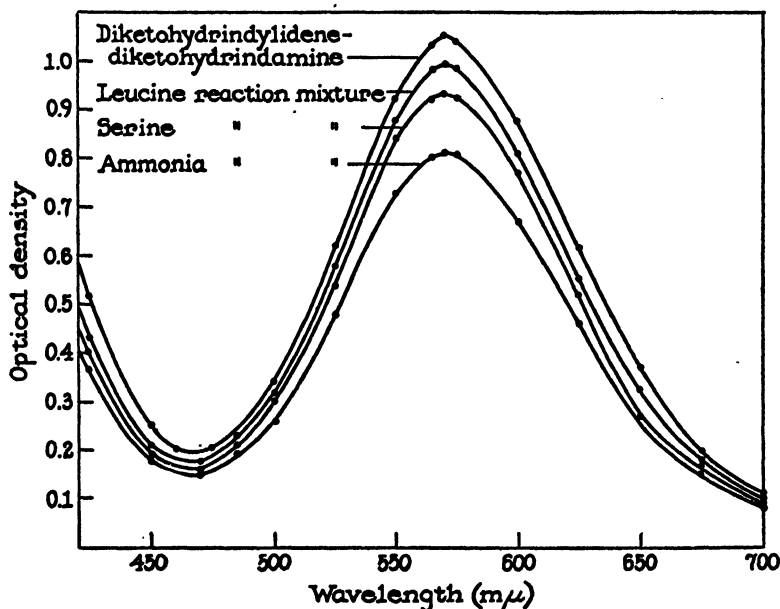


FIG. 2. Comparison of the absorption spectrum of diketohydrindylidene-diketohydrindamine with the spectra obtained after the reaction of ninhydrin with equimolar amounts of leucine, serine, and ammonia.

under the conditions of the determination. The curves obtained with the other α -NH₂ acids, except cysteine, and with peptides, are similar to those shown in Fig. 2. The major end-product is the same in all cases, exhibiting an absorption maximum at 570 mμ. The differences in the color intensities obtained with the individual amino acids arise from variations in the yield of this product. Relative to leucine, the reading of the pure sodium salt corresponds to a color yield of 1.07 (Table II). On this basis, leucine yields about 93 per cent of the theoretical amount of this product. The yields for phenylalanine and glutamic acid, for example, are 82 and 98 per cent.

Color development with ninhydrin is not specific for amino acids, since a variety of primary amines and some secondary amines will give significant amounts of color. The structure of the end-products in these cases remains to be determined. The absorption curves obtained with histamine and with the N-methyl-substituted amino acid, sarcosine, are given in

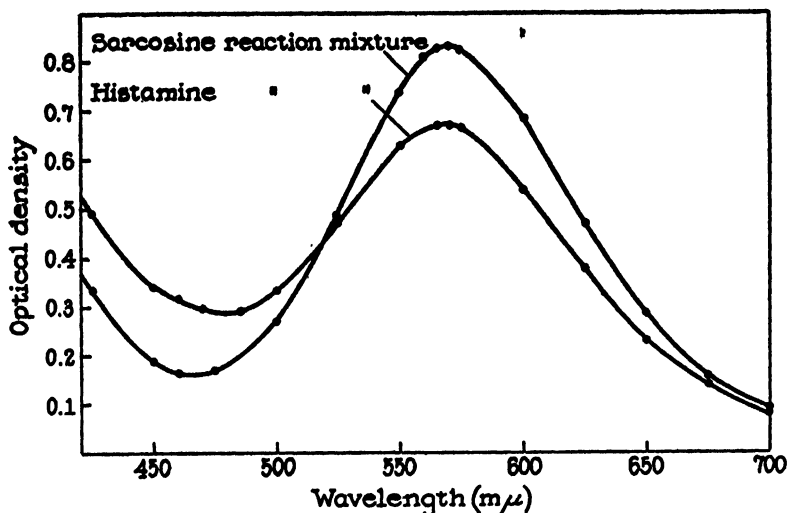


FIG. 3. Absorption spectra obtained after the reaction of ninhydrin with sarcosine and histamine.

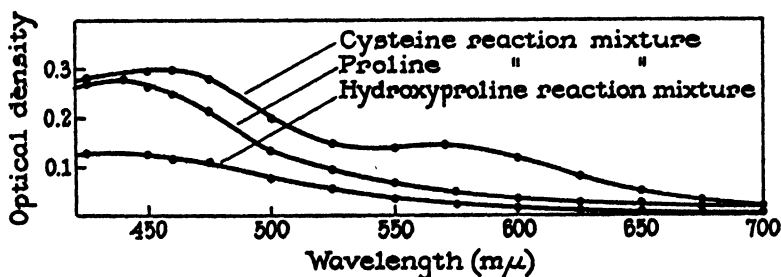


FIG. 4. Absorption spectra obtained after the reaction of ninhydrin with proline, hydroxyproline, and cysteine.

Fig. 3. Both compounds yield products with absorption maxima at 570 mμ.

Cysteine, which has been mentioned as an exception to the general reaction of the α -NH₂ acids, gives an absorption curve which is somewhat similar to that obtained with proline and hydroxyproline (Fig. 4). Neutral cysteine solutions, after standing for 24 to 48 hours, give the same color

yield as cystine, with maximum absorption at $570\text{ m}\mu$. It is possible that the small amount of absorption in the range of $570\text{ m}\mu$ observed with fresh solutions of cystine may be attributed to the presence of some cystine in the reaction mixture. The compounds yielded by the prolines under these conditions presumably correspond to the structures assigned by Grassmann and von Arnim (12).

Variation of pH—The variation of color yield with the pH of the aqueous citrate buffer is shown in Fig. 5. The absorption maximum for the $\alpha\text{-NH}_2$ acids is at $570\text{ m}\mu$ over the pH range studied. The maximum color yield from leucine is obtained at pH 5, which has been chosen for the general procedure.

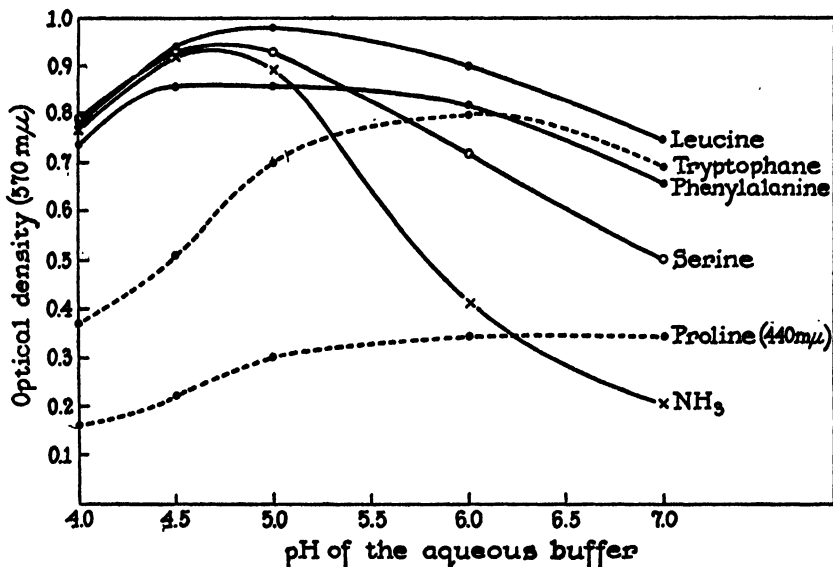


FIG. 5. Effect of pH on the intensity of color obtained after the reaction of ninhydrin with amino acids and ammonia.

The pH optima for the other $\alpha\text{-NH}_2$ acids fall close to this value, with the exception of tryptophan which gives a maximum yield at pH 6. For most of the amino acids, a change in pH of 0.1 unit at pH 5.0 introduces less than a 1 per cent deviation in the optical density reading.

Rate of Reaction—The rates of color development have been determined for representative $\alpha\text{-NH}_2$ acids over a range of pH from 4 to 7. In all cases, at 100° the reaction was complete in less than 20 minutes. The color yields were unaltered by an increase in the ninhydrin concentration. This result parallels the observations on the heating periods required for the complete liberation of CO_2 from amino acids at a ninhydrin concentration of 20 mg. per cc. in the gasometric method of Van Slyke, Dillon, Mac-

Fadyen, and Hamilton (18). A more detailed study of the rate of color development has been made at pH 5. Constant readings are obtained with leucine in 5 minutes and with alanine in 10 minutes. The reaction with glycylphenylalanine and glycylleucine is complete in 20 minutes. On the other hand, phenylalanylglycine and leucylglycine reach only 90 per cent of completion in this time. Primary amines, such as ethylamine and ethanolamine, react still more slowly. The time of 20 minutes has been chosen as a heating period which gives constant readings with all of the α -NH₂ acids and can be expected to give reasonably high readings with most peptides possessing a free NH₂ group.

Temperature—The color yields are lower if the reaction is carried out at temperatures below 100°. For leucine, isoleucine, tyrosine, and phenylalanine, the optical densities were 4 per cent lower when the analysis was carried to completion in a water bath maintained at 95°.

Stability of Color—The rate of fading of the blue color is illustrated by the following average readings obtained on leucine samples at the specified times after removal of the photometer tubes from the heating bath: 15 minutes 0.835, 30 minutes 0.830, 45 minutes 0.835, 60 minutes 0.835, 1½ hours 0.815, 2½ hours 0.810, 4 hours 0.795, 5½ hours 0.785, and 22 hours 0.710. Thus, the color is stable for about 60 minutes, after which time there is a gradual fading, averaging approximately 1 per cent per hour. The end-product is not highly sensitive to oxidation by air, whereas, as noted below, an intermediate in the reaction appears to possess much greater sensitivity.

In early experiments, water was used as the diluent and marked fading was noted as a result of precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine. The use of 1:1 water-*n*-propanol as the diluent serves to keep the relatively insoluble reaction product in solution.

Effect of Stannous Chloride—When 2 mm leucine samples are analyzed with a ninhydrin solution from which the stannous chloride has been omitted, the color yield is about half that obtained in its presence. At lower leucine concentrations the percentage decrease in yield is greater. By carrying out the reaction in vessels evacuated to 20 to 30 mm., as is done in the gasometric ninhydrin method (13), the color yield from 2.0 mm leucine solutions can be raised almost to the maximum value. At a leucine concentration of 0.05 mm, however, the results still run about 10 per cent low.

The first trials on the blocking of the oxidative side reaction by the addition of a reducing agent to the ninhydrin solution were made with hydrindantin. Consistent results were obtained at a hydrindantin concentration of 1 mg. per cc. The color yields were unaltered by a 4-fold increase in this concentration. Since hydrindantin can be prepared by the action

of stannous chloride on ninhydrin, the addition of stannous chloride directly to the reagent solution was tried and found to give the same results. However, the presence of hydrindantin, which is highly insoluble in water, required the addition of an organic solvent which would keep this compound in solution during the course of the reaction and during storage of the reagent solution. Among the solvents tested, methyl cellosolve had the highest solvent power for hydrindantin. The solvent mixture chosen (1:1 water-methyl cellosolve) does not evaporate in the water bath at 100° and does not precipitate sodium citrate from the buffer.

SUMMARY

The reaction of ninhydrin with NH_2 groups to give diketohydrindylidene-diketohydrindamine has been utilized as the basis for a photometric determination of amino acids and related compounds in effluent samples from starch chromatograms. The color yields have been rendered fully reproducible by the incorporation of hydrindantin or stannous chloride in the reagent solution to eliminate oxidative side reactions. Although the color yield from a given amino acid is constant, the different amino acids do not all give the same percentage yield of the blue product. This fact does not prevent the accurate use of the method in chromatographic work in those cases in which the individual amino acids are separated from one another by the fractionation process.

Color development is obtained with a variety of compounds containing NH_2 groups, including amino acids, peptides, primary amines, and ammonia. For chromatographic work, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity is a disadvantage.

The reaction is carried out at pH 5 and 100°. The absorption maximum of the blue product is at 570 μ . On individual amino acids the accuracy is 2 per cent for samples in the range of 2.5 γ of $\alpha\text{-NH}_2$ nitrogen. The mechanics of the procedure have been developed to permit the analysis of a large number of samples on a routine basis.

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THE ACONITE ALKALOIDS

XXI. FURTHER OXIDATION STUDIES WITH ATISINE AND ISOATISINE

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The mild oxidation of isoatisine, $C_{22}H_{33}O_2N$, with permanganate to a neutral derivative, $C_{22}H_{33}O_3N$, has been previously described (1). Further studies have shown that isoatisine and atisine can be oxidized to a mixture of acidic substances by more drastic procedures and the separation of several of these acids is reported here. The oxidation presumably passes through the neutral lactam stage such as oxoisoatisine, since the acids are not amphoteric and have given no indication of the retention of a basic N group.

Isoatisine has given a *tricarboxylic acid*, $C_{21}H_{29}O_7N$, in good yield. The additional oxygen is assumed to be present in the lactam grouping. The acid melts with evolution of approximately 1 M equivalent of CO_2 (within 15 minutes). Since the acid was stable to heating with rather concentrated acid or alkali, decarboxylation of a malonic or acetoacetic acid type appears excluded. Both the acid and its thorium salt were pyrolyzed under a variety of conditions in attempts to obtain a cyclic ketone, the formation of which might have been the source of the CO_2 . However, only small amounts of a resinous mixture resulted, from which nothing crystalline could be obtained. The study of such material was therefore inconclusive, although its neutralization equivalent indicated the retention of approximately two carboxyl groups.

The *trimethyl ester* obtained with diazomethane from the acid, $C_{21}H_{29}O_7N$, although non-crystalline, was purified by sublimation. It could be saponified quickly to a crystalline *monomethyl ester*. The latter proved to be relatively resistant to further saponification. The carboxyl group involved appears most probably to be of tertiary character. The monomethyl ester when heated above its melting point only very gradually yielded an appreciable amount of CO_2 , and after an hour this amounted to about 0.5 M equivalent. This suggests that the tertiary carboxyl group may be the source of the CO_2 in the case of the acid itself.

The tricarboxylic acid when refluxed with aniline yielded a derivative, $C_{27}H_{33}O_5N_2$, titration of which showed it to be a monocarboxylic acid. The compound must therefore be a cyclic *N-phenylimide* which indicates the proximity of the two carboxyl groups involved. On treatment of the

phenylimide with diazomethane, the imide ring was opened with the formation of a *dimethyl ester anilide*.

In an attempt to hydrogenate the tricarboxylic acid in methanol which contained HCl, hydrogenation did not occur but the substance recovered proved to be a *dimethyl ester*. In the latter, because of its ease of saponification to the original tricarboxylic acid, the tertiary carboxyl group could not have been involved. The dimethyl ester, however, now appeared sufficiently stable on heating to permit of its distillation unchanged.

The tricarboxylic acid was dehydrogenated with selenium to a mixture of phenanthrene hydrocarbons from which 1,6-dimethylphenanthrene was separated. If one excludes the unlikely production of a phenanthrene hydrocarbon by ring closure of a naphthalene fragment or the rather improbable conversion of a carboxyl to a methyl group during the dehydrogenation, the production of 1,6-dimethylphenanthrene is of special significance, since it has already been shown that 1-methyl-6-ethylphenanthrene is a dehydrogenation product of atisine (2). This indicates that in the production of the acid, $C_{21}H_{23}O_7N$, from isoatisine, the oxidation must have involved that portion of the molecule which gave rise to the ethyl group in methyl-ethylphenanthrene.

The probable absence of a double bond in the tricarboxylic acid was indicated by the failure of attempts to hydrogenate it in methanol, in acetic acid, or in methanolic HCl. On such a basis, the formulation requires its tetracyclic character. Other evidence is against its unsaturated tricyclic character. If the latter were the case, the non-basic lactam N atom would be restricted to a side chain. But all attempts to hydrolyze the substance proved futile. It was recovered unchanged in excellent yield after treatment with 8 N HCl at 150° for 6 hours, after solution in concentrated H_2SO_4 at room temperature for several days, after refluxing in 10 per cent NaOH for 6 hours, after fusion with molten alkali at 300°, and after treatment with the methyl Grignard reagent under forcing conditions. A simple amide linkage would be expected to be opened by such procedures. The observed stability of this linkage may be due to its inclusion in the fourth ring of the tricarboxylic acid, with a steric arrangement which is very resistant to cleavage.

An effort was made to determine whether the nitrogen was of secondary or of tertiary character. The substance was treated with bromine in methanolic NaOH under the conditions of the Hofmann degradation but was recovered unchanged. Similarly unsuccessful were attempts to brominate it in neutral or in acid solution. Because of the negative outcome of such experiments, the degree of substitution of the lactam nitrogen has not been determined. It appears certain, however, that the latter does not bear an ethyl group, since the 21 carbon atoms of the substance have been accounted

for in the following manner: 16 are present in the 1,6-dimethylphenanthrene fragment, 3 in the carboxyl groups, 1 in the lactam group, and 1 in the carbon atom lost during the oxidation of isoatisine to the $C_{21}H_{29}O_7N$ acid. Although the original alkaloid gave about 50 to 60 per cent of the required amount in the N-alkyl determination, oxoisoatisine gave about 35 per cent and the tricarboxylic acid only about 19 per cent of the theoretical for N ethyl (determined as NCH_3). Isoatisine under the conditions of the N-alkyl determination has previously been shown to yield ethyl iodide together with small amounts of methyl iodide (1). But the difficulties which we have encountered in the N-alkyl determinations with this class of substances have made it unsafe to base too much on the results obtained. There is a possibility that if atisine and isoatisine do not possess a simple N-ethyl group, the tertiary N could be contained in two rings in such a way that the source of the ethyl iodide is a two carbon bridge involving the N atom. The loss of two carbon fragments has already been indicated by the conversion of oxoisoatisine with methanolic HCl to an apparent $C_{20}H_{29}O_2N$ derivative and by the production of a $C_{20}H_{29}ON$ compound from atisine by catalytic dehydrogenation under high pressure (1). But the available data do not permit a final decision as to whether the so called N-ethyl group is involved in these transformations.

More recently, an apparent loss of a two carbon fragment accompanying partial decarboxylation of the tricarboxylic acid has also been noted. By treatment of the latter with either thionyl chloride or bromine and phosphorus tribromide followed by decomposition with water, a crystalline *dicarboxylic acid* was produced for which a possible formulation, $C_{15}H_{25}O_6N$, has been suggested by the analytical data.

The tricarboxylic acid does not appear to be a monosubstituted acetic acid, RCH_2COOH , because of the failure to brominate it under the conditions of the Hell-Volhard-Zelinsky method and because of its recovery unchanged when the Hunsdiecker reaction (3), *i.e.* the action of bromine on its silver salt, was attempted.

Atisine, when oxidized under the same conditions used for isoatisine, yielded a *dicarboxylic acid*, $C_{21}H_{29}O_6N$, in very small yield, together with oxalic acid. No oxalic acid was isolated from the oxidation of isoatisine described above. The acid which was separated from the oxidation mixture by the counter-current procedure of Craig (4) showed no basic properties and was therefore of lactam character. It proved to be stable above its melting point and could be sublimed unchanged. The saponification of its crystalline *dimethyl ester* to a more resistant *monomethyl ester* suggests the tertiary character of one of the carboxyl groups. The substance absorbed hydrogen in acetic acid and, although a crystalline product was obtained, its formulation remains uncertain and will be left to a later communication.

Like the tricarboxylic acid from isoatisine, this acid, from its formulation and ability to react with hydrogen, possibly contains four rings. It did not react with hydroxylamine and its absorption spectrum did not indicate the presence of a carbonyl group. By a more drastic oxidative procedure with permanganate, a $C_{15}H_{27}O_8N$ tricarboxylic acid was also obtained from atisine. It was isolated in very small yield by the counter-current procedure.

From the facts at hand it is still premature to attempt to suggest definite structures for atisine and isoatisine. However, the data appear to permit of certain conclusions. There appears to be present a pentacyclic structure made up in part of a perhydrophenanthrene nucleus with a methyl group attached to position 1. Fused to this is a bicyclic structure containing the tertiary N atom. One point of attachment of this moiety to the perhydrophenanthrene nucleus appears to be at position 6; in isoatisine the hydroxyl groups and the two double bonds of the original alkaloids do not appear to be a part of the perhydrophenanthrene nucleus. One of the hydroxyl groups appears to be of a primary character and the source of a carboxyl group on oxidation. A ring which is outside of the perhydrophenanthrene nucleus and contains a double bond, and perhaps one of the hydroxyl groups, appears to be ruptured during the oxidation to the tricarboxylic acid.

EXPERIMENTAL

Tricarboxylic Acid, $C_{15}H_{27}O_8N$, from Isoatisine—5 gm. of isoatisine in 100 cc. of benzene were rapidly stirred with a solution of 1.25 gm. of NaOH in 100 cc. of water and cooled to 10°. At this temperature 15 gm. of powdered $KMnO_4$ were added during 3 hours. An additional 5 gm. were added and the stirring continued overnight to complete the oxidation. The benzene was separated by centrifugation and yielded a negligible amount of resinous material. After removal of the MnO_2 , the aqueous filtrate was strongly acidified with HCl. A doughy mass separated which was reduced to a powder on chilling and collected. After solution in a minimum of hot water and cooling, platelets separated. By concentration two successive fractions were obtained. The total yield was 2.6 gm. It decomposed at 258–261° with gas evolution.

$$[\alpha]_D^{25} = +9.3^\circ \text{ (c = 1.34 in methanol)}$$

$C_{15}H_{27}O_8N$.	Calculated.	C 61.88,	H 7.18,	N 3.44,	(N)CH ₃ 3.68
	Found.	(a) " 62.04,	" 7.04,	" 3.45,	" 0.70
		(b) " 61.63,	" 7.40,	" 3.50	

Neutralisation equivalent (3COOH), calculated 135.7, found 139

176 mg. of the acid were heated in a sublimation apparatus at 300° for 15 minutes while a slow stream of CO_2 -free air was passed through the apparatus into a tower packed with glass beads containing 0.1 N $Ba(OH)_2$.

Very soon the production of CO_2 was evident. The temperature was lowered to 120° and the air stream was continued for 30 minutes. Titration with 0.1 N HCl showed that 0.85 equivalent of CO_2 had been produced. An attempt was made to sublime the residue at 0.05 mm. from a bath at 300° but only 15 mg. of an amber resin were obtained.

100 mg. of the acid when melted and sublimed as rapidly as possible at 320° and 0.05 mm. yielded 35 mg. of a resin, C 62.20, H 7.03. A second experiment gave a resin which yielded C 62.93, H 7.50.

Various methods of fractionation yielded nothing crystalline from such material. When the resin was dissolved in a large volume of ether and concentrated, an amorphous powder separated. Analysis showed C 64.23, H 7.51; neutralization equivalent 176.

Methyl Esters of Tricarboxylic Acid—0.2 gm. of the $\text{C}_{21}\text{H}_{29}\text{O}_7\text{N}$ acid dissolved in methanol was treated with an excess of diazomethane in ether. After removal of the solvent, the resinous residue of the trimethyl ester could not be crystallized and was sublimed at 300° and 0.05 mm. 130 mg. of a resin were obtained.

$$[\alpha]_D^{25} = +9.6^\circ \text{ (c = 2.09 in ethanol)}$$

$\text{C}_{24}\text{H}_{33}\text{O}_7\text{N}$.	Calculated.	C 64.10, H 7.85, OCH_3 20.71
	Found.	" 64.43, " 7.89, " 19.61

187.3 mg. of the trimethyl ester were heated at 100° in a mixture of 5 cc. of methanol and 13 cc. of 0.157 N NaOH. 0.5 cc. aliquots were withdrawn at intervals and back-titrated with 0.02 N acid. After 5, 30, and 100 minutes, the alkali consumed was 1.89, 2.06, and 2.13 equivalents. The solution was concentrated to a small volume and acidified. The resinous mass which separated crystallized on boiling. After recrystallization from water, 62 mg. of rod-shaped crystals of the monomethyl ester of the acid were obtained, which melted at $210\text{--}215^\circ$.

$$[\alpha]_D^{25} = +1.8^\circ \text{ (c = 1.67 in ethanol)}$$

$\text{C}_{22}\text{H}_{31}\text{O}_7\text{N}$.	Calculated.	C 62.67, H 7.42, N 3.33, OCH_3 7.36, $(\text{N})\text{CH}_3$ 3.56
	Found.	" 62.46, " 7.48, " 3.48, " 7.15 " 0.72

Neutralization equivalent (2COOH), calculated 210.6, found 222

220 mg. of the monomethyl ester were heated in the apparatus as described above with the tricarboxylic acid itself. The substance melted at about 200° . Since no BaCO_3 was observed, the temperature was raised to 280° in 15 minutes, at which point a slight precipitate of BaCO_3 became evident. Heating was continued at this temperature for 60 minutes as the BaCO_3 slowly increased. By titration it was shown that 0.52 M equivalent of CO_2 had been produced. The residue, when subjected to a sublimation at 280° and 0.05 mm., yielded 70 mg. of a non-crystallizable resin. Found, C 64.53, H 8.12.

A rapid sublimation was performed by melting 200 mg. of the ester at 220° and then rapidly raising the temperature to 300° with reduction of the pressure to 0.02 mm. 170 mg. of a resin were obtained. Found, C 63.01, H 7.38. From dilute methanol 80 mg. were obtained crystalline; m.p. 200–210°. The melting point of a mixture with the original substance was 208–215°.

$C_{22}H_{31}O_7N$. Calculated, C 62.67, H 7.42; found, C 62.48, H 7.52

2.1 gm. of the tricarboxylic acid were shaken for 3 days with 0.60 gm. of platinum oxide catalyst in 50 cc. of methanol which contained 2 cc. of HCl (1.19). The hydrogen absorption did not exceed that required by the catalyst. The substance recovered from the mixture proved to be a dimethyl ester and crystallized from methanol-water as stout rhombs which softened at 235° and melted at 240–245°.

$[\alpha]_D^{25} = +15.3^\circ$ (c = 2.02 in ethanol)

$C_{22}H_{31}O_7N$. Calculated. C 63.41, H 7.64, OCH_3 , 14.26

Found. " 63.55, " 7.68, " 13.80

Neutralization equivalent, calculated, 435.27, found 410

200 mg. of the dimethyl ester were sublimed at 250° and 0.02 mm. and the sublimate was recrystallized from dilute methanol. 130 mg. of starting material were recovered which melted at 240–245°.

209 mg. of the dimethyl ester were heated at 100° with 14 cc. of 0.157 N NaOH. Samples were withdrawn after 5, 15, and 60 minutes and showed the consumption respectively of 2.95, 2.95, and 2.99 equivalents of alkali. After acidification of the mixture the tricarboxylic acid was recovered and melted with decomposition at 260–261°.

Aniline Derivatives of Tricarboxylic Acid and Ester—100 mg. of the $C_{21}H_{29}O_7N$ acid were refluxed in 0.5 cc. of freshly distilled aniline for 30 minutes. Cold dilute HCl was added to the mixture and the solid material was collected. It was decomposed with dilute NaOH and the excess aniline was extracted with ether. The alkaline phase was acidified with HCl and the resulting gum was extracted with ethyl acetate. The extract after concentration yielded material which partially crystallized from ethanol-water. After repeated recrystallization from the same solvent, 30 mg. of stout needles were obtained which softened at 293° and melted at 299–302° with gas evolution.

$C_{27}H_{35}O_4N_3$. Calculated. C 69.79, H 6.95, N 6.03

Found. " 69.92, " 6.98, " 6.30

Neutralization equivalent, calculated 464.26, found 476

20 mg. of the aniline derivative were suspended in 2 cc. of methanol and an excess of diazomethane in ether was added. After an hour at room

temperature the solvents were removed and the residue was dissolved in ethyl acetate. The solution was shaken with 5 per cent NaOH, dried, and evaporated to dryness. The residue yielded from dilute methanol 15 mg. of fine needles which melted at 230–235°. After three recrystallizations, the melting point was 235–238°.

$C_{21}H_{23}O_7N_2$. Calculated. C 68.19, H 7.50, N 5.49, OCH₃ 12.16
Found. " 68.32, " 7.30, " 5.83, " 10.98

Dehydrogenation of Tricarboxylic Acid—A mixture of 2 gm. of the acid $C_{31}H_{29}O_7N$ and 2.3 gm. of selenium was heated in a tube fitted with an air condenser. At 260° vigorous gas evolution accompanied by water condensation was noted. The temperature was raised to 320° for 7 hours. The ground reaction mass was repeatedly extracted with hot benzene. The benzene solution was shaken in turn with 10 per cent HCl and 5 per cent NaOH. The former was made alkaline with NaOH and extracted with ether. The latter yielded on concentration 50 mg. of a basic fraction. The NaOH extract after acidification with HCl yielded a negligible amount of acidic material. The benzene solution of the neutral fraction is described below.

The crude basic fraction was chromatographed on alumina (Merck) from benzene. By elution with benzene a zone which showed blue fluorescence under ultraviolet light was obtained. It consisted of 10 mg. of a colorless oil with a quinoline-like odor. The picrate was prepared and, after two recrystallizations from ethanol, 5 mg. of small yellow needles were obtained which melted at 311–316°. Further study of the substance has been deferred because of the amount available.

$C_{17}H_{21}N \cdot C_6H_5O_7N_2$. Calculated, C 58.69, H 5.57; found, C 58.75, H 5.74

The benzene solution of the neutral fraction when concentrated to dryness yielded 0.51 gm. of a brown tar. This was chromatographed on alumina from benzene. On elution with benzene a band which fluoresced under ultraviolet light emerged and amounted to 0.11 gr. This was rechromatographed on alumina from petroleum ether. Elution with 10 per cent benzene in petroleum ether yielded 50 mg. of a colorless oil which crystallized on chilling. After two recrystallizations from ethanol, 20 mg. of plates were obtained which softened at 90° and melted completely at 105°. Since it was an obvious mixture, its further fractionation was studied.

A chromatograph on alumina with petroleum ether as solvent was prepared. The column was developed and eluted with 1:10 benzene-petroleum ether. As a broad fluorescing zone emerged, it was divided into two

fractions. The first fraction after recrystallization from ethanol melted at 85–95°.

$C_{18}H_{14}$. Calculated, C 93.15, H 6.85; found, C 93.31, H 6.78

The second fraction yielded crystals which melted at 95–100°.

Found, C 93.32, H 6.63

The picrate of the first fraction was prepared and after two recrystallizations from ethanol melted at 140–141°. The hydrocarbon was regenerated

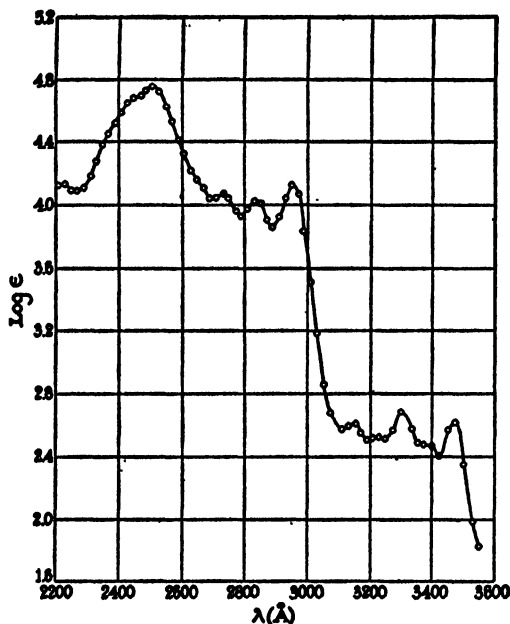


FIG. 1. $C_{18}H_{14}$ hydrocarbon in ethanol

from the picrate by passing its ethanol solution through alumina. After recrystallization from ethanol, the hydrocarbon was obtained as plates which melted at 84–88°. The ultraviolet absorption spectrum is shown in Fig. 1. A sample of 1,6-dimethylphenanthrene, which was kindly sent to us by Professor R. D. Haworth, melted at 88–90°. A mixture of the two melted at 86–88°. The picrate of the purified dehydrogenation hydrocarbon crystallized from ethanol in yellow-orange needles which melted at 142–143°.

$C_{18}H_{14} \cdot C_6H_5O_7N_3$. Calculated. C 60.66, H 3.93
 Found. " 60.57, " 3.81

The melting point of a mixture with the picrate prepared from the authentic 1,6-dimethylphenanthrene was 142–143°. The trinitrobenzene addition product prepared from the hydrocarbons from both sources crystallized from ethanol in pale yellow needles and melted at 163–164°. No depression in melting point was noted with a mixture of the two substances.

Action of Bromine-Phosphorus Tribromide and of Thionyl Chloride on Tricarboxylic Acid—2 gm. of the tricarboxylic acid were mixed with 1 cc. of bromine, and 0.5 cc. of phosphorus tribromide was added with stirring. After standing overnight at room temperature, the mixture was heated on the steam bath for 1 hour and then boiled with 50 cc. of water. On cooling, a yellow resin formed. This was recrystallized in portions from water as described below for the thionyl chloride product. The substance separated as small aggregates of microscopic often triangular leaflets which melted at 220–223°.

$C_{15}H_{25}O_6N$. Calculated. C 64.44, H 7.52, N 4.18

$C_{15}H_{27}O_6N$. " " 64.05, " 8.07, " 4.15

Found. " 64.40, " 7.95, " 4.13

Neutralization equivalent, calculated 167.6, 168.6, found 185

150 mg. of the substance in methanol were treated with an excess of diazomethane. After removal of the excess reagent and solvent by concentration the residue was sublimed at 200° and 0.02 mm. 130 mg. of a resin were collected which could not be crystallized.

$C_{20}H_{39}O_6N$. Calculated. C 66.07, H 8.05, OCH_3 17.08

$C_{20}H_{41}O_6N$. " " 65.71, " 8.55, " 17.00

Found. " 65.68, " 8.18 " 17.05

0.1 gm. of the tricarboxylic acid, when stirred with 1 cc. of purified thionyl chloride for 30 minutes, gradually dissolved. After warming to 60° for 10 minutes, excess reagent was removed *in vacuo*. The residue was boiled with 100 cc. of water and filtered from a yellow gum. The extract was concentrated to about 10 cc., when triangular leaflets separated which were larger than in the previous case. 30 mg. were collected. They lost birefringence at 200° and melted at 220° with decomposition.

Found. (a) C 64.11, H 7.97, N 3.91

(b) " 64.15, " 7.84

Neutralization equivalent, found 188

Methylation with diazomethane and distillation at 220° and 0.02 mm. yielded a resin which could not be crystallized and closely resembled in properties the ester described above. Found, C 65.78, H 8.39.

Attempted Degradation of Tricarboxylic Acid with Bromine (3)—200 mg. of the tricarboxylic acid were dissolved in an equivalent of dilute NaOH

and treated with a solution of 0.27 gm. of silver nitrate in 3 cc. of water with vigorous shaking. The gelatinous silver salt was collected, washed well with water, and then dried *in vacuo* over phosphorus pentoxide. The resulting 300 mg. were refluxed in 20 cc. of dry carbon tetrachloride and a solution of 0.2 M bromine in this solvent was added. 10 cc. were rapidly decolorized. An additional 10 cc. were added and the heating was continued for 30 minutes. The solid material was filtered off and the filtrate on evaporation yielded a negligible residue. The silver salt was decomposed with dilute HCl and in turn shaken with dilute NaOH. The filtrate from the solid material when acidified with HCl yielded a gum which was collected. On recrystallization from water 90 mg. of plates were obtained which melted with decomposition at 259–262° and proved in other ways to be unchanged tricarboxylic acid.

$C_{21}H_{23}O_7N$. Calculated, C 61.88, H 7.18; found, C 61.47, H 7.05

Dicarboxylic Acid, $C_{21}H_{23}O_8N$, from *Atisine*—5 gm. of *atisine* hydrochloride were stirred rapidly in a mixture of 100 cc. of benzene and 100 cc. of H_2O which contained 1.25 gm. of NaOH. The temperature was kept at 10° and 8.6 gm. of $KMnO_4$ were added in small portions during 3 hours. The mixture was then allowed to reach room temperature and an additional 9.4 gm. of $KMnO_4$ were added in three portions. By morning the reagent had been completely used up. The aqueous and benzene phases were separated and the latter yielded only 50 mg. of a neutral resin which was discarded. The aqueous filtrate was acidified with HCl and 3.2 gm. of a resin were collected. The filtrate from this was concentrated to dryness and the residue was extracted repeatedly with hot ethyl acetate. The latter on slow concentration yielded large crystals. These were recrystallized from water. 0.23 gm. of oxalic acid dihydrate was obtained. The latter lost crystal water at 105–109° and decomposed with vigorous gas evolution at 195–196° and agreed in other properties with an authentic sample.

Neutralization equivalent, calculated 63.02, found 63.2

The 3.2 gm. of resin were subjected to a nine stage counter-current distribution in separatory funnels with, in each case, 50 cc. of ethyl acetate and 50 cc. of 2 M phosphate buffer (pH 5.9). After the distribution each funnel was made strongly acid with H_2SO_4 and repeatedly extracted with ethyl acetate. The material from Funnels 5 and 6 partially crystallized after evaporation of the solvent. The crystalline material was collected with ethyl acetate and recrystallized from dilute ethanol. 0.2 gm. of rhombs was obtained which melted at 323–326° after preliminary softening at 310°.

$$[\alpha]_D^{25} = -23.5^\circ \text{ (c = 1.82 in ethanol)}$$

$C_{21}H_{21}O_6N$. Calculated. C 64.41, H 7.47, (N)CH₃ 3.84

Found. (a) " 64.60, " 7.33 " 1.12

" (b) " 64.37, " 7.41

Neutralisation equivalent, calculated (2COOH) 195.62, found 193

After recrystallization from an ethanol-ethyl acetate-petroleum ether mixture, the melting point remained essentially unchanged.

Found, C 64.29, H 7.57

100 mg. of the acid were sublimed at 250° and 0.02 mm. 80 mg. of a partially crystalline resin were collected. Found C 64.37, H 7.60. When recrystallized from ethyl acetate it melted at 325–330°, and showed no depression when mixed with the original acid. Found C 64.30, H 7.41.

Dimethyl Ester and Monomethyl Ester—100 mg. of the dicarboxylic acid in acetone suspension were treated with excess diazomethane. After removal of solvent the residue was recrystallized from dilute ethanol. It formed needles; m.p. 193–195°.

$C_{23}H_{21}O_6N$. Calculated. C 65.83, H 7.93, OCH₃ 14.80

Found. " 65.72, " 7.89, " 14.20

30.6 mg. of the dimethyl ester dissolved in 2 cc. of methanol were heated at 100° with 2 cc. of 0.157 N NaOH. Aliquots withdrawn after 1 hour and 1½ hours showed respectively the consumption of 0.99 and 1.04 equivalents of alkali. The solution was acidified and concentrated to small volume. Needles separated which were collected and recrystallized from dilute ethanol; m.p. 229–232°.

$C_{23}H_{21}O_6N$. Calculated. C 65.14, H 7.71, OCH₃ 7.65, (N)CH₃ 3.70

Found. " 65.07, " 7.70 " 7.30, " 0.73

" 65.56, " 7.72

C₁₉H₁₇O₆N Acid from Atisine—5 gm. of atisine hydrochloride were stirred with a solution of 1.5 gm. of NaOH in 50 cc. of H₂O and 50 cc. of benzene. Without cooling 30 gm. of KMnO₄ were added in small portions during 2 hours. The aqueous phase was then separated. This was heated on the steam bath and an additional 10 gm. of KMnO₄ were added during 2 hours. The filtrate from MnO₂ was acidified with HCl. The filtrate from a small gummy precipitate was extracted three times with 25 cc. portions of ether which on evaporation yielded 1.07 gm. of a syrup. Continued ether extraction of the aqueous phase for 3 days yielded a mixture of crystals and some syrup. The crystals were collected with ether. After recrystallization from water 80 mg. of oxalic acid dihydrate were obtained which lost water at 105–109° and melted with decomposition at 195–196°.

The above 1.07 gm. of syrup were subjected as described above to a nine-stage Craig counter-current distribution between 50 cc. phases of ether and 2 M phosphate buffer (pH 5.3) in separating funnels. After the distribution each funnel was acidified (pH 3) with phosphoric acid and the aqueous phase was extracted repeatedly with ether. Each fraction was then examined. The material from Funnels 4, 5, and 6 partially crystallized on removal of the ether. That from Funnel 5 was collected with ether and, when recrystallized from ethanol-ether, yielded 15 mg. of very small prisms. The substance softened at 205°, then resolidified in a different crystalline form and again melted at 310–315°. From Funnels 4 and 6, 25 mg. of the same substance were obtained. The melting point of a mixture of this substance with the above dicarboxylic acid, $C_{21}H_{29}O_6N$, from atisine showed a definite depression (m.p. 285–293°).

	$[\alpha]_D^{25} = +39^\circ$ ($c = 1.08$ in ethanol)
$C_{19}H_{27}O_6N$.	Calculated. C 62.43, H 7.45, N 3.84, (N)CH ₃ 4.11
	Found. " 62.35, " 7.14, " 3.82, " 0.00
Neutralization equivalent, calculated (3COOH) 121.74, found 127	

All analytical data have been obtained by Mr. D. Rigakos of this laboratory.

SUMMARY

A further study of the oxidation of isoatisine with permanganate has yielded a tricarboxylic acid, $C_{21}H_{29}O_7N$, which also contains the lactam grouping. The tertiary character of one of its carboxyl groups has been suggested by the behavior of the methyl esters with alkali. Dehydrogenation of this acid has yielded 1,6-dimethylphenanthrene which conforms with the production of 1-methyl-6-ethylphenanthrene from atisine itself.

Further study of the oxidation of atisine with permanganate has yielded a dicarboxylic acid, $C_{21}H_{29}O_6N$, and a tricarboxylic acid, $C_{19}H_{27}O_6N$.

From the data obtained certain tentative conclusions have been drawn in regard to a number of structural features of atisine.

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THE VERATRINE ALKALOIDS

XXVIII. THE STRUCTURE OF JERVINE

BY WALTER A. JACOBS AND YOSHIO SATO

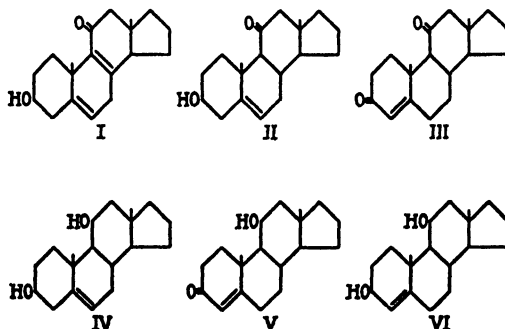
(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, April 14, 1948)

Since the previous communication (1) on jervine, new observations have been made which, with reinterpretation of some of the earlier data, now permit of certain conclusions in regard to its structure. Most satisfactory, as presented in Formula I, is that of a 3(β)-hydroxy-11-keto- $\Delta^4,8(9)$ -steroidal base in which the secondary basic group is contained on the iso-octyl side chain. The probable oxidic linkage appears also to be attached at least in part to the side chain but its exact location remains to be determined.

In the course of the earlier work to identify the double bond still present in dihydrojervine the attempt was made to convert this derivative, as with jervine itself, with aluminum tert-butoxide to the Δ^4 -ketone and the latter in turn with aluminum isopropoxide to a possible Δ^4 -dihydrojervine. The last stage yielded a number of fractions of a crystalline hydrochloride which gave no Rosenheim test or only a weak one with trichloroacetic acid. At the time this appeared to support the possibility of hydrogenation of the Δ^8 double bond of jervine in the formation of dihydrojervine. However, other evidence has since been accumulated which makes this untenable. Much more clear cut was the experience with β -dihydrojervinol (Formula IV) in which the carbonyl group of dihydrojervine has been reduced with sodium and butanol (1). The dihydrojervine (Formula II) used for reduction to β -dihydrojervinol was first treated with HCl to remove contaminating jervine by isomerization. β -Dihydrojervinol was smoothly converted by the Oppenauer method to the crystalline Δ^4 -3-keto derivative $C_{27}H_{41}O_2N$ (Formula V) which readily yielded an *oxime*. The ultraviolet absorption spectrum obtained with the Δ^4 -ketone is shown in Fig. 1, together with that of the original β -dihydrojervinol. The ketone was in turn readily reduced with aluminum isopropoxide to the crystalline Δ^4 -3-hydroxy derivative $C_{27}H_{43}O_2N$ which, contrary to β -dihydrojervinol, now gave a pronounced Rosenheim reaction (Formula VI). No attempt has been made as yet to determine the configuration of the 3-hydroxyl group of the substance isolated or to separate possible epimers, if a mixture. However, the product was isolated in several fractions which closely agreed in properties.

This series of reactions parallels the experience with jervine itself and with other 3-hydroxy- Δ^5 -steroids. It has been assumed that the original carbonyl group of jervine as such, or after its reduction with sodium to hydroxyl, has not participated in these reactions. This was especially indicated by the general unreactivity of the carbonyl group of jervine. Aside from its reduction with sodium, this inertness was shown by the failure of all more recent attempts to cause it to react with ketone reagents. Similarly jervine was recovered unchanged after the usual treatment with aluminum isopropoxide. This behavior strongly indicates a CO group at position 11 (2, 3). As a possible additional check for the 11-keto group, the behavior was studied of the ease of acylation of the OH group formed from it by reduction. However, β -dihydrojervinol in pyridine at room temperature with acetic anhydride yielded quite readily *N*-acetyldihydrojervinol diacetate. As in the case of sarmentogenin this ease of acylation



indicates a less hindered configuration for the new hydroxyl group (3) than that found with the adrenal 11-hydroxy steroids (2).

In the earlier paper absorption spectrum studies with dihydrojervine were discussed. Due to the abnormal results obtained, the possibility of contamination with unchanged jervine was considered but discarded because of the failure of repeated attempts to change these results by certain purification procedures. However, this has since been found to be incorrect by the use of a relatively large amount of catalyst for the first step in the hydrogenation of jervine. A dihydrojervine was obtained with properties essentially as previously recorded with the exception, however, that the ultraviolet absorption data obtained with it (Fig. 1) now no longer showed the strong absorption in the shorter wave-lengths. The curve closely resembles that previously obtained from dihydrojervine which had been recovered after treatment with HCl to remove possible contamination with jervine. However, the presence of some absorption in the shorter wave-lengths suggests the persistence of slight contamination. The peak which persists at about $300\text{ m}\mu$ is due to carbonyl group absorption.

In view of the normal behavior of β -dihydrojervinol, we returned to a study of dihydrojervine in which the same acid-treated material was used. With aluminum tert-butoxide and acetone a product resulted which was purified through alumina and gave in good yield a rather low melting ($131-132^\circ$) crystalline substance. The ultraviolet absorption spectrum of the latter (Fig. 1), contrary to that of dihydrojervine, now yielded the absorption to be expected of Δ^4 -dihydrojervone as shown in the partial Formula III.

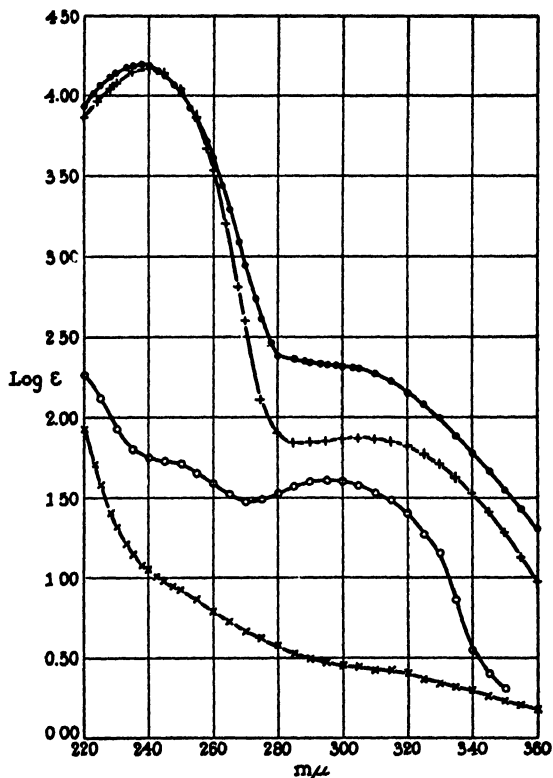


Fig. 1. ○ = dihydrojervine; ● = Δ^4 -dihydrojervone; + = Δ^4 - β -dihydrojervinol; × = β -dihydrojervinol; all in ethanol.

The latter was further characterized by its *oxime*. The next step with aluminum isopropoxide, however, again gave ambiguous results. The reaction product isolated as the base in a number of crystalline fractions failed in largest amount to give the expected Rosenheim test. Only a very small fraction was obtained which gave a sufficiently strong, although abnormal, color with trichloroacetic acid. In addition, analytical discrepancies (0.5 to 1 per cent high in C) persisted in attempts to characterize

the various fractions. Because of these difficulties a report of the experimental details of this part of the investigation will be deferred, pending the outcome of a further study planned to explain the anomalous results.

In the course of our work a personal communication was received from D. H. R. Barton who correctly questioned the earlier abnormal ultraviolet absorption curve attributed to dihydrojervine (and since rejected by us) as being due to a $\Delta^{\alpha,\beta}$ -ketonic type of absorption. He also called attention to previously published data of this laboratory (1) as follows: If one corrected for possible contamination of the dihydrojervine used with about 4 per cent of jervine, its molecular rotation would be -341° . Since that observed with tetrahydrojervine was -90° , the observed change in molecular rotation was $+251^\circ$ on passing from the former to the latter. Similarly from our data the hydrogenation of β -dihydrojervinol with its molecular rotation of -17° to tetrahydrojervinol of molecular rotation $+211^\circ$ shows a change of $+228^\circ$. Both of these results approximate the change in molecular rotation of $+240^\circ$ shown by Barton (4) to be characteristic for the hydrogenation of a Δ^5 -steroid bond.

We have also found that the expected 3(β)-hydroxyl as well as the Δ^5 -stenol character of jervine which does not form an insoluble digitonide has been confirmed by the application of the method of Barton (5) to *N*-acetyljervine ($[\alpha]_D^{27} = -118.5^\circ$ ($c = 1.07$ in chloroform)). A comparison of the molecular rotation of the latter with those of *N*-acetyljervine acetate, $[\alpha]_D^{27} = -114^\circ$ ($c = 1.08$ in chloroform), and *N*-acetyljervine benzoate, $[\alpha]_D^{27} = -81^\circ$ ($c = 0.96$ in chloroform), has shown that the molecular rotations are respectively -553° , -580° , and -463° , or a Δ_1 of -27° and a Δ_2 of $+91^\circ$. The Δ_1 and Δ_2 values of Barton are respectively -35° and $+81^\circ$.

The persistence of the Δ^5 bond in β -dihydrojervinol (and also in α -dihydrojervinol) shows that this bond in dihydrojervine and jervine resists reduction with sodium and thus conforms to the behavior of the usual 3-hydroxy- Δ^5 -steroids first shown with cholesterol itself (6, 7).

In earlier work it was found that the highly hydroxylated tertiary bases cevine (8), germine (9), and protoverine (10) are reduced with sodium to dihydro bases. Since this differs from the usual behavior for a Δ^5 -stenol, a recent check was made on the behavior of rubijervine, isorubijervine, and solanidine. As was to be expected, they were not reduced by this method.¹ A further difference between the cevine group and the simpler bases as already recorded was the resistance of the former to the usual hydrogenation procedure (except in the case of cevine with nickel). However, after isomerization with alkali to iso bases (9, 10), presumably due

¹ The statement previously made ((1) p. 639, paragraph 2) conveys an incorrect and unintended meaning in this regard and should be discarded.

to a bond shift, such hydrogenation readily occurred. It is probable that these natural polyhydroxy bases are $\Delta^{8(14)}$ -stenols and the iso bases are Δ^{14} derivatives. A suggestion of this was seen by the use of the method of molecular rotation differences of Barton (11).

On passing from isogerminine of $[\alpha]_D^{25} = -46.5^\circ$ ($c = 1.01$ in 95 per cent ethanol) to the value recently found for dihydroisogerminine of $[\alpha]_D^{27} = -51.4^\circ$ ($c = 1.01$ in 95 per cent ethanol), a Δ of -26° is obtained which agrees with the Δ value of -24° given by Barton for a Δ^{14} -stenol (β -stenol). A similar result, *viz.* $\Delta = -27^\circ$, is obtained by using the rotations of these substances found in pyridine respectively of $[\alpha]_D^{28} = -56^\circ$ ($c = 1.00$) and $[\alpha]_D^{28} = -61^\circ$ ($c = 0.95$).

With isoprotoverine and dihydroisoprotoverine (10), because of sparing solubility, the previously reported rotations were taken only in pyridine. A more recent value for the former also in this solvent was $[\alpha]_D^{28} = -44^\circ$ ($c = 0.94$) and the rotation for dihydroisoprotoverine recalculated for the dried substance was $[\alpha]_D^{28} = -53^\circ$. From these figures the value obtained for Δ is -48° . Although not as close as in the case of isogerminine to the above Δ value, it also suggests a β -stenol type for isoprotoverine.

As a final point, the identity of the groups involved in the isomerization of jervine with acid to isojervine becomes of interest. Previously (1) the possibility of the formation of a new hydroxyl group by cleavage of the oxidic grouping was discussed. However, the stability of dihydrojervine to acid and the ready acetylation of the new hydroxyl group of isojervine to form an N-acetylisojervine diacetate (1) suggest that isojervine may result from the enolization of the 11-CO group and conjugation of the new double bond to form a possible benzenoid Ring B. Although the latter is supported by the resistance of isojervine to hydrogenation, its previously published ultraviolet absorption curve does not conform to a simple benzenoid type. The study of this question will therefore be continued.

EXPERIMENTAL

Δ^4 - β -Dihydrojervonol—For the material used in this study 6 gm. of dihydrojervine obtained by the hydrogenation of jervine were treated with methanol (75 cc.) saturated with HCl at 0° and the paste of hydrochloride was allowed to come to room temperature. After an hour the salt was collected with a small amount of methanol and then decomposed with aqueous alkali for extraction with chloroform. After removal of solvent the dihydrojervine was crystallized from methanol. This material was reduced as previously described in butanol with sodium. The β -dihydrojervinol obtained after recrystallization melted sharply at 283.5 – 286° .

1.8 gm. were refluxed with 7.2 gm. of aluminum tert-butoxide dissolved

in a mixture of 170 cc. of benzene and 60 cc. of acetone for about 4 hours. After decomposition with dilute alkali the product was extracted with chloroform. The resin obtained by concentration of the washed and dried extract was dissolved in 50 cc. of acetone and allowed to stand for several days. 0.19 gm. of β -dihydrojervinol which crystallized was recovered. The mother liquor on concentration to 10 cc. yielded a crust of heavier crystals. After several hours this was collected with cold acetone. 0.9 gm. was obtained which sintered at 211° and melted at 219 – 223° . It separated without solvent as micro platelets on addition of ether to the benzene solution and after slight preliminary sintering melted at 221.5 – 223.5° .

$C_{37}H_{41}O_3N$. Calculated, C 75.82, H 9.67; found, C 75.56, H 9.42

The *oxime* was obtained in methanol solution with hydroxylamine hydrochloride and sodium acetate. The diluted mixture was made alkaline and extracted with chloroform. The oxime separated from 50 per cent methanol as flat micro needles which lost birefringence at 286 – 292° .

$C_{37}H_{43}O_3N_2$. Calculated, C 73.24, H 9.57; found, C 73.57, H 9.50

Δ^4 - β -Dihydrojervinol—0.3 gm. of Δ^4 - β -dihydrojervinol was dissolved in benzene and the solution was concentrated *in vacuo* to dryness to insure removal of all solvent. The residue was treated with a solution of aluminum isopropoxide in 25 cc. of isopropanol prepared from 1 gm. of aluminum. After refluxing for 2 hours, an additional 15 cc. of isopropanol were added and the mixture was very slowly distilled until the distillate no longer gave a Legal test. The mixture was further concentrated to small volume and, after cooling and decomposition with excess dilute NaOH, it was extracted with chloroform. The latter yielded on concentration a resin which was dissolved in methanol followed by careful dilution. When crystallization once began it was greatly aided by warming and formed a crust of small platelets. This fraction of 90 mg. on recrystallization from dilute methanol formed almost rhombic wedge-shaped aggregates of micro platelets which melted at 246 – 248° and were anhydrous. It gave a gradually developing pronounced deep purple color with trichloroacetic acid.

$[\alpha]_D^{25} = +54.5^\circ$ ($c = 1.03$ in chloroform)
 $C_{37}H_{43}O_3N$. Calculated. C 75.46, H 10.10
 Found. (a) " 75.16, " 10.09
 (b) " 75.81, " 10.14

The mother liquor of the above fraction contained most of the material and, although further dilution caused crystallization, it was found preferable to reextract with chloroform. The concentrated chloroform solution on addition of benzene caused a copious crystallization of aggregates of

pointed platelets which contained 1 mole of solvent. This fraction (0.16 gm.) melted at 243–246° and gave a Rosenheim test indistinguishable from the first fraction. For analysis it was dried at 110° and 0.2 mm.

$[\alpha]_D^{25} = +66^\circ$ ($c = 0.80$ in chloroform for dry substance)

$C_{17}H_{44}O_2N \cdot C_6H_6$. Calculated, C 15.38; found, 14.97

$C_{17}H_{44}O_2N$. Calculated, C 75.46, H 10.10; found, C 75.67, H 10.10

The *hydrochloride* separated from methanol ether as aggregates of micro platelets which sintered progressively above 235° and melted at 272–275° (uncorrected).

For analysis it was dried at 110° and 0.2 mm.

$C_{17}H_{44}O_2NCl$. Calculated, C 69.56, H 9.52; found, C 69.63, H 9.57

Δ^4 -Dihydrojervone—0.5 gm. of dried dihydrojervine was refluxed for 5.5 hours with 1.2 gm. of aluminum tert-butoxide in a mixture of 18 cc. of benzene and 9 cc. of acetone. At first almost clear, the mixture gradually showed considerable deposit which later became somewhat colored. After dilution and treatment with excess alkali, the mixture was extracted with chloroform. The washed and dried extract was concentrated and then repeatedly concentrated with benzene and, finally, *in vacuo*. However, the residue remained an oil. This was dissolved in 5 cc. of benzene and chromatographed at first with this solvent through 12 gm. of an active alumina. When material began to appear it was collected in 5 cc. portions. The first few fractions yielded an appreciable oily residue which smelled of acetone condensation products but from Fraction 5 yielded crystalline material. After Fraction 12 the solvent was changed to 0.5 per cent methanol in benzene to facilitate the otherwise very gradual elution. Following an interpretation of the general course of the elution and ease of crystallization, Fractions 26 to 38 inclusive were joined. On concentration *in vacuo* 0.28 gm. of a resin was obtained. On careful dilution of the acetone solution it gradually yielded a copious mass of needles which were collected with acetone-water (1:2). 0.195 gm. was obtained which melted at 122–128°. After recrystallization from dilute acetone it formed aggregates of needles which contained little solvent and melted sharply at 131–132°. It was readily soluble in the usual organic solvents.

$C_{17}H_{32}O_2N$. Calculated, C 76.18, H 9.24; found, C 76.62, H 9.37

From later fractions, especially after changing to 1 per cent methanol, a very small amount of unchanged dihydrojervine was recovered.

The *oxime* was prepared by refluxing the methanol solution of 28 mg. with 60 mg. of hydroxylamine hydrochloride and 0.2 gm. of sodium acetate. After 3 hours the concentrated solution was treated with excess ammonia and diluted before the sparingly soluble hydrochloride could separate.

The oxime separated as delicate needles. 24 mg. were collected. It tended to separate from methanol or diluted methanol as a gelatinous precipitate. From the warm diluted solution it crystallized as micro leaflets which lost birefringence at 293–297°.

$C_{27}H_{40}O_2N_2$. Calculated, C 73.58, H 9.15; found, C 73.30, H 8.98

N-Acetyl- β -dihydrojervinol Diacetate—0.1 gm. of β -dihydrojervinol was refluxed in 3 cc. of acetic anhydride for 3 hours. After removal of excess reagent the residue crystallized from methanol without solvent as micro platelets or needles which melted at 254–257°.

$C_{23}H_{34}O_4N$. Calculated, C 71.30, H 8.89; found, C 71.20, H 8.90

A suspension of 75 mg. of β -dihydrojervinol in 0.7 cc. of pyridine and 0.35 cc. of acetic anhydride was shaken at room temperature until dissolved and left for 20 hours. After repeated concentration *in vacuo* with benzene the residue was dissolved in methanol. From small volumes at 0° successive fractions of 28, 21, and 30 mg. were obtained. The first fraction (a) melted sharply at 256–257° while the second fraction (b) melted at 235–237°. The third fraction (c) melted again at 255–256°.

Found. (a) C 71.41, H 8.98

(b) " 71.51, " 9.06

(c) " 71.28, " 8.90

N-Acetyljervine Benzoate—0.1 gm. of *N*-acetyljervine (12) in 2 cc. of pyridine was treated in the cold with 0.1 cc. of benzoyl chloride. After 2 hours it was poured into excess cold dilute H_2SO_4 . After hardening, the product was collected. It formed rosettes of needles from 95 per cent ethanol which, although free from solvent, appeared to melt partly at 159–163° and then at 218–223°. When taken a few days later, it melted only at 219–223°.

$C_{28}H_{40}O_2N$. Calculated, C 75.61, H 7.94; found, C 75.58, H 7.71

All microanalytical work was performed by Mr. D. Rigakos of this laboratory.

SUMMARY

New observations with reinterpretation of previous data now permit of certain conclusions in regard to the structure of jervine. Most satisfactory is that of a 3(β)-hydroxy-11-keto- $\Delta^{5,8(9)}$ -steroidal secondary base in which the basic group is contained on the isooctyl side chain. The probable oxidic linkage appears also to be attached at least in part to the side chain.

There is evidence that the highly hydroxylated tertiary veratrine bases cevine, germine, and protoverine are $\Delta^{8(14)}$ -stenols and that their iso derivatives are Δ^{14} derivatives.

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A BALL-BEARING DRIVE FOR THE ULTRACENTRIFUGE

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The authors have built and tested a stable ball-bearing drive for the ultracentrifuge. The drive has been incorporated into a Pickels¹ turbine, with the addition of Beams, Linke, and Sommer's² speed control. The use of the air lift in the air-driven centrifuge has been accompanied by axial oscillations, caused by small fluctuations in the air pressures. To minimize this effect Pickels utilized an oil damping device in his turbine. The present design eliminates the oscillations completely by providing a stable and simple bearing and improves the focus of the optical system. A schematic cross section of the turbine is shown in Fig. 1, and a photograph of the mechanism in Fig. 3. The novel part of the apparatus, i.e., the ball-bearing arrangement, is given in Fig. 2 which is enlarged from Fig. 1 and carries the same lettering. The apparatus includes a thrust bearing, *C* (New Departure Model No. 0104), having a single race and five balls, which is located beneath the impeller, *B*. Contact between the balls and the impeller is made through a small hardened and concentrically ground cone, *N*, which projects from the under surface of the impeller. A screw, *K*, locks the cone both to the impeller and to the drive shaft, *M*, through pressure which is exerted by it on the collet, *L*. The diameter of the cone *N* at the plane of contact with the balls, about $\frac{3}{16}$ ", is the same as that of one of the balls. Thus the speed of rotation of the balls is the same as that of the impeller. An Oilite bronze bearing, *D*, serves as a guide for the shaft and contributes oil to the ball bearing. To minimize the axial thrust on the bearing an air lift is used. Six pounds per square inch of lift pressure is almost sufficient to raise the impeller off the balls with the rotor in place. The balls, the race, and the cone showed no apparent wear after 200 hours operation at 54,000 to 61,000 r.p.m.

In the speed control method of Beams, Linke, and Sommer² the rotation of the magnet *F* between the poles of the field core *G* produces an alternating current in the coils *E* wound on this core. These coils are connected to an external condenser. The energy absorbed by the coil and condenser circuit increases sharply as the resonance frequency is approached, furnishing opposition to further increases of speed. A guide bearing, above the magnet, consists of two bronze bearings, *H*, pressed into a bronze ball, *I*, which is free to align itself in the housing and is held by two Neoprene rings, *O*. A small

¹ E. G. Pickels, Rev. Sci. Inst. 9, 358 (1938).

² J. W. Beams, F. W. Linke, and P. Sommer, Rev. Sci. Inst. 18, 57 (1947).

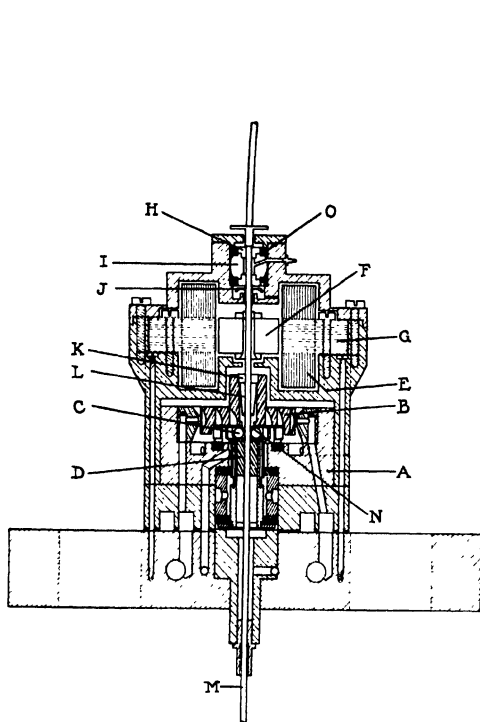


FIG. 1

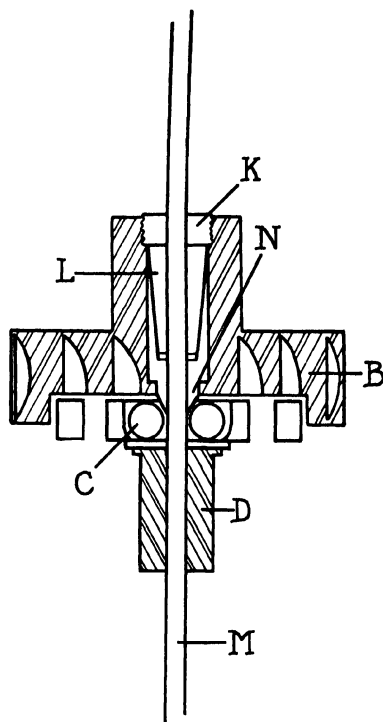


FIG. 2

FIG. 1. Schematic cross section of the turbine.
 FIG. 2. Arrangement of the ball-bearing assembly in the turbine.

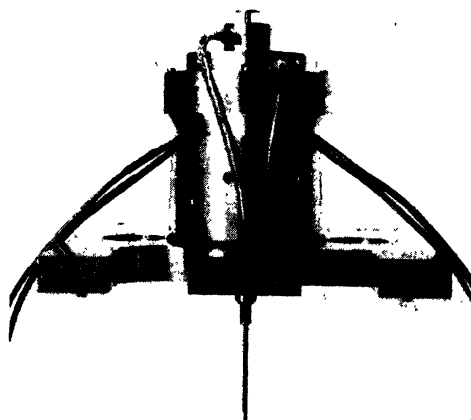


FIG. 3. The assembled turbine.

aluminum disk, J , throws off the oil which leaks by the bearing. This oil is collected in a trap milled into the housing.

The increase in bearing surface in the turbine as well as the added mass of the magnet have lengthened the time of acceleration of this centrifuge about 20 percent. It has been found necessary to maintain a driving pressure on the impeller of from 60 to 61 pounds per square inch to operate the turbine on the steepest part of the resonance curve for this circuit where a large change in driving pressure results in a small change in speed. The speed may be controlled to 0.1 percent at 1000 r.p.s.

The authors wish to express their thanks to Dr. D. A. MacInnes of this laboratory for his continued interest and advice in this project, and to Dr. J. W. Beams of the Rouss Laboratory of Physics, University of Virginia, for his help in the design and construction of the speed control.

MAGNESIUM PROTOPORPHYRIN AS A PRECURSOR OF CHLOROPHYLL IN CHLORELLA*

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In this paper, we wish to describe the separation and identification of another intermediate in chlorophyll synthesis from another *Chlorella* mutant.

The *Chlorella vulgaris* mutant 60 was isolated as an orange-colored colony after irradiation of normal green *Chlorella* cells with x-rays. Single cells from this colony showed constancy in their properties. The colonies which developed on the inorganic salts-glucose agar medium had a dull yellow color which turned orange-brown in 4 to 7 days when grown either in the light or dark at room temperature. At 36° growth was good but the colonies remained dull yellow. At 15° growth was very poor and colonies also remained dull yellow. The cells were grown in large flasks and harvested after 7 to 8 days. To minimize the chances of dealing with a mixed population due to spontaneous mutation, cultures for a large batch were always started from a typical colony derived from a single cell.

Extraction of Pigments from Supernatant Suspension of Cells—The cells were found to contain a complex mixture of pigments, the predominating ones being the carotenoids, with small amounts of protoporphyrin, magnesium protoporphyrin, and traces of a greenish pigment. By shaking the cells in distilled water, a reddish brown cloudy suspension was obtained which was relatively free of carotenoids and had absorption bands at 640, 590, 540 to 550, 470 to 480, and 420 to 425 mμ, as measured in a Beckman spectrophotometer (Fig. 1). Pigments from this cloudy suspension were readily isolated by treating the suspension with an equal volume of alcohol, saturating with NaCl, and extracting into ether. Preliminary tests showed that protoporphyrin was present in the ether solution. To get rid of the protoporphyrin the ether solution was washed with water several times and then rapidly extracted with an equal volume of ice-cold 1 N HCl.¹ (The acid aqueous layer was shown spectrophotometrically to contain protoporphyrin.) The ether layer was immediately treated with an equal

* This is the fourth of a series of papers on porphyrins and related compounds. For the third paper, see Granick (1).

¹ If no foaming occurs, then only a little Mg protoporphyrin will be decomposed by shaking the ether layer with cold 1 N HCl.

volume of ice-cold 1:1 solution of 0.02 N KOH and absolute alcohol. A pinkish fluorescent pigment entered the aqueous phase. (The residue in the ether now consisted of carotenoids and of a trace of a greenish pigment.)

The absorption spectrum of the aqueous alcohol solution was measured and the solution was found to contain two components (Fig. 2). The major component had two prominent bands in the visible with maxima at 550 and 588 $m\mu$ and an intense ultraviolet maximum at 418 $m\mu$; this com-

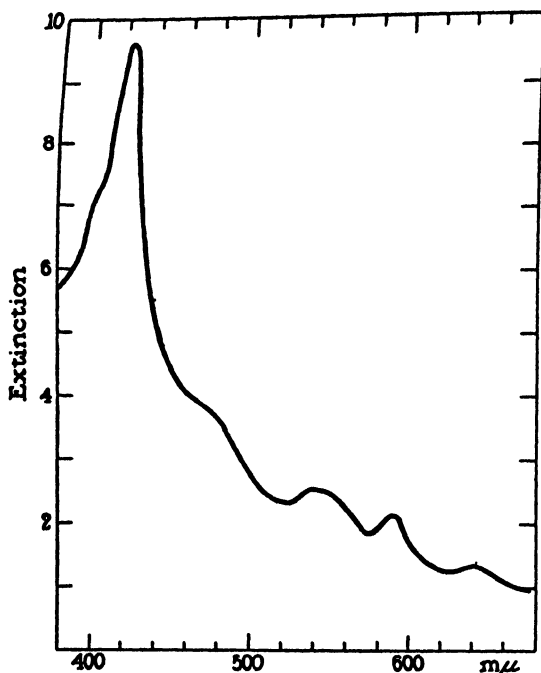


FIG. 1. Absorption spectrum of cloudy supernatant from cells, where extinction signifies observed densities.

ponent was later identified as magnesium protoporphyrin. The other, lesser component was found to be protoporphyrin which explained the bands at 630, 530, and 505 $m\mu$. The bands seen in the cloudy aqueous suspension (Fig. 1) are best interpreted as representing colloidal aggregates of protoporphyrin with a small amount of magnesium protoporphyrin (1).

Extraction of Pigments from Cells—The isolation of the pigment, having bands at 550 and 588 $m\mu$, was difficult because of the sensitivity of this pigment to acids, its low concentration, and the rather large amounts of contaminating yellow pigments. No simple procedure was found. Only

the general principles of the isolation will be described: it was necessary to control each step of the isolation by observations in the hand spectroscope.

To keep the cells slightly alkaline in order to avoid splitting out of the Mg, sodium bicarbonate was added to some 100 cc. of packed cells, and these were extracted until colorless with 80 per cent alcohol and 80 per cent acetone. To remove yellow pigments and fats and traces of green pigments, the alcoholic solutions were diluted with an equal volume of water, made alkaline with NH_4OH , and extracted with ether. The aqueous solution was brought to pH 5.5 to 6.0 with solid KH_2PO_4 and shaken with

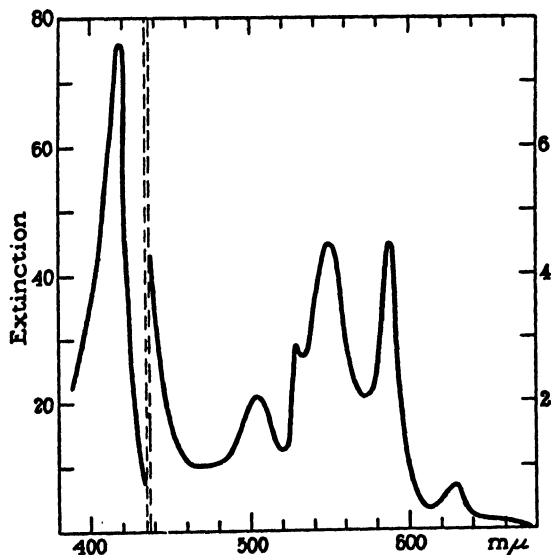


FIG. 2. Absorption spectrum of alkaline alcoholic solution derived from supernatant. The three prominent bands are due to magnesium protoporphyrin. The extinction is 10 times higher on the left side than on the right side. The extinction signifies the observed densities.

a small volume of *n*-amyl alcohol. Overnight in the ice box, the pigment was found to have collected in the amyl alcohol layer. The amyl alcohol layer was separated and evaporated to dryness under reduced pressure; the pink pigments were taken up in ether and shaken into a 50 per cent alcoholic layer containing dilute NH_4OH and again driven into ether by acidifying cautiously with acetate buffer and saturating the aqueous alcoholic layer with NaCl . This transfer between ether and aqueous alcohol was repeated twice more. The ether solution was then concentrated to 10 cc. The ether solution now contained as major components the two pigments, protoporphyrin and Mg protoporphyrin. By shaking

the ether solution with 0.05 cc. of 3 N NH_4OH , and then placing in the ice box overnight, the protoporphyrin was found to be precipitated out at the interface. (Under these conditions Mg protoporphyrin precipitated out only after 3 to 4 days.)

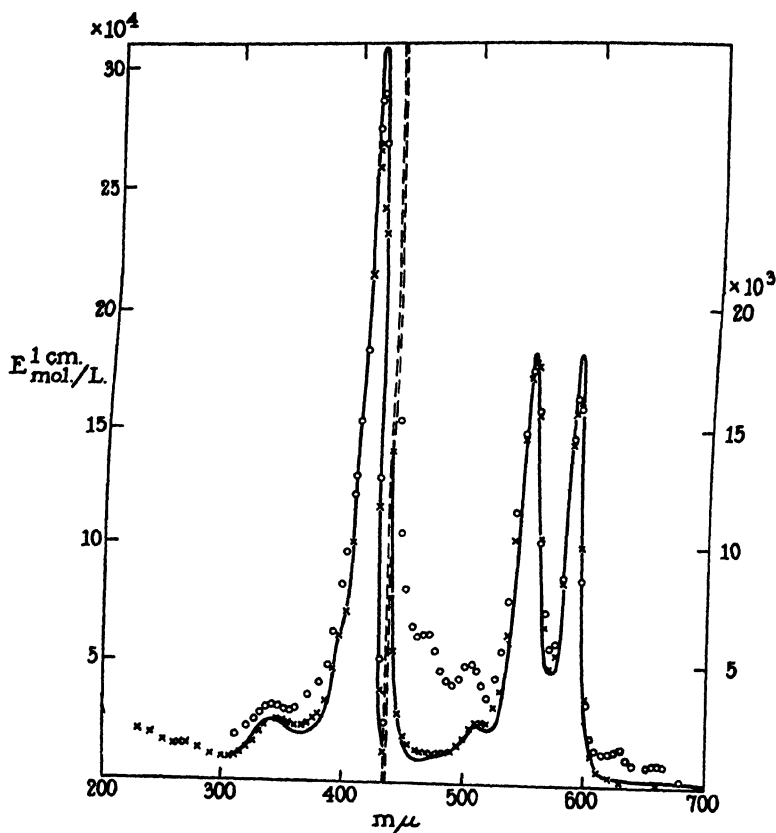


Fig. 3. Smooth curve, absorption spectrum of synthetic Mg protoporphyrin dimethyl ester in ether. X, absorption spectrum of synthetic Mg protoporphyrin in 0.02 N KOH containing 50 per cent ethanol. O, absorption spectrum of pigments isolated from *Chlorella* 60, measured in 0.02 N KOH containing 50 per cent ethanol; the extinction values were adjusted at 588 $m\mu$ to the Mg protoporphyrin curve by a factor and the remaining points were multiplied by this factor.

An aliquot of this solution, evaporated to dryness, was taken up in a solution of 0.02 N KOH containing 50 per cent ethyl alcohol and measured spectrophotometrically. One point on this absorption curve (*i.e.* 588 $m\mu$) was adjusted to the curve for synthetic Mg protoporphyrin by a factor, and the remaining points were multiplied by this factor (Fig. 3). From

Fig. 3 it is seen that the predominating pigment in this solution corresponds to synthetic Mg protoporphyrin with respect to the positions of the absorption maxima (418, 551, and 589 $m\mu$) and the relative heights of the bands. The solution is still contaminated by small amounts of protoporphyrin, as seen in the bands at 505 and 630 $m\mu$, possibly by carotenoids (*i.e.* band at 465 $m\mu$), and by a trace of a greenish pigment (640 to 670 $m\mu$). Since the solution was estimated to contain only about 0.5 mg. of Mg protoporphyrin, further purification by chromatographing was not attempted.

Identification of Porphyrin of Metal Complex As Protoporphyrin—Since only about 0.5 mg. of the magnesium protoporphyrin was isolated from *Chlorella*, and since the isolation in the crystalline state would have been too tedious, it was deemed necessary to obtain supporting evidence for the composition of this compound by identification of the kind of porphyrin and the kind of metal. An aliquot of the ether solution was extracted with 3 N HCl. At this acidity the metal was split off and all of the pink pigment entered the aqueous phase. The aqueous solution was neutralized and the porphyrin reextracted into ether. The ether solution was washed with water and then extracted successively with increasing concentrations of HCl. No porphyrins were extractable from ether with HCl solutions below 0.1 N. Two fractions were isolated by extraction between 0.1 and 0.4 N HCl and between 0.4 and 1.0 N HCl. The absorption spectra of both these fractions fell, within experimental error, on the curve of pure protoporphyrin (Fig. 4). This result indicates that neither a monovinyl nor any other porphyrin except protoporphyrin was present, and therefore the pigment originally isolated must be a derivative of protoporphyrin. Neither esters of magnesium protoporphyrin nor esters of protoporphyrin could be found in this preparation or in crude preparations that had been extracted from cells in which the use of alkaline fluids was avoided. Such esters would have been detected in the ether after extraction with 1 N HCl. (When *Chlorella* 60 was grown in a medium containing 1 mg. of Cu per liter, a small amount of pigment was observed which was stable in strong HCl; the positions of the band maxima were those of Cu protoporphyrin.)

Identification of Magnesium As Metal in Complex—The qualitative identification of magnesium was carried out with the quinalizarin reagent (2). The test is highly specific, only beryllium and lanthanum besides magnesium being reported to form a blue precipitate in strongly alkaline solution. However, the test is not particularly sensitive. A method was devised to remove the porphyrin, which interferes with observation of the blue precipitate, and at the same time to keep the magnesium as concentrated as possible.

The test was carried out in the following way: 5 cc. of the ether solution, estimated to contain approximately 250 γ of magnesium protopor-

phyrin, were placed in a 15 cc. conical centrifuge tube. Then 0.10 cc. of 4.6 N HCl was added, and air was bubbled through to stir the HCl into the ether layer. After several minutes the ether layer became completely colorless and all of the protoporphyrin was now collected into the drop of aqueous HCl at the bottom of the tube. The HCl was neutralized by adding 0.12 cc. of 4.00 N NaOH plus 0.01 cc. of glacial acetic acid to the tube. Air was again bubbled through until all of the protoporphyrin had passed back into the ether layer. The tube was then centrifuged. The clear colorless aqueous droplet at the bottom of the tube was now removed

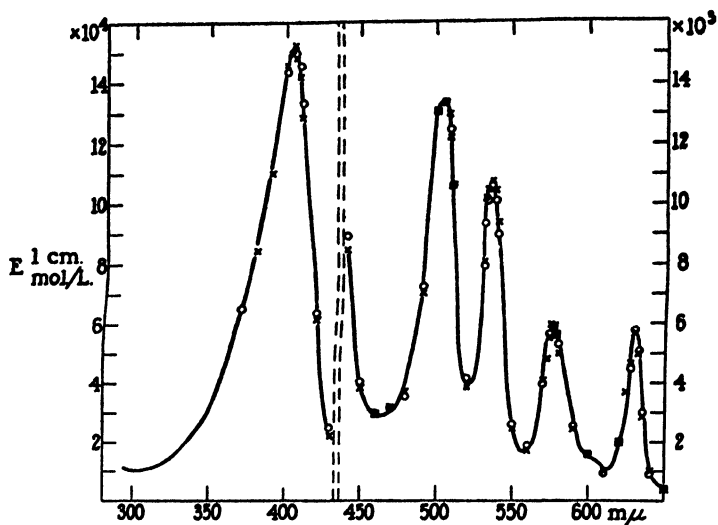


FIG. 4. Smooth curve, absorption spectrum of synthetic protoporphyrin IX in ether. Absorption of porphyrin derived from magnesium protoporphyrin isolated from mutant *Chlorella*; ×, porphyrin extracted between 0.1 and 0.4 N HCl; O, porphyrin extracted between 0.4 and 1.0 N HCl. The extinction is 10 times higher on the left side than on the right side.

with a capillary pipette and placed on a drop plate, and 2 drops of alcoholic quinalizarin (10 mg. per cent) and 2 drops of 2 N NaOH were added. Blue granules appeared within 1 minute. At the same time and on the same drop plate a series of known concentrations of Mg^{++} was run, including controls of the reagents. From the rate at which the blue granules appeared and their volume it was estimated that the quantity of Mg^{++} was about 10 γ, which was in the predicted range if the compound was Mg^{++} protoporphyrin. (At this concentration Ca^{++} does not give a characteristic blue precipitate.)

Neither magnesium protoporphyrin nor its ester has ever been prepared

in the crystalline form. It was deemed necessary to prepare the magnesium protoporphyrin and to study its properties, in order to compare it with the pigment derived from the *Chorella* mutant. The dipotassium salt of magnesium protoporphyrin was made by way of the dimethyl ester.

Preparation of Magnesium Protoporphyrin Dimethyl Ester—The method used here, of inserting the magnesium into protoporphyrin by means of a decomposed Grignard reagent, is a modification of that used by Fischer and Dürr (3). To obtain this compound in its crystalline form, it was found necessary to use highly purified protoporphyrin ester, to avoid temperatures above 80°, and to run the reaction preferably in the absence of O₂.

In a 500 cc. round bottom triple necked flask was placed 1.0 gm. of Mg ribbon. This was washed by decantation with anhydrous ether. Then 30 cc. of anhydrous ether and 15 cc. of ethyl bromide were added. The flask was connected to a reflux condenser with a drying tube attached, and the contents warmed gently. After some 20 minutes the reaction was ended. To distil off the ether and excess ethyl bromide most easily, the water in the reflux condenser was emptied, and the flask, still attached to the condenser, was placed in hot water. Toward the end of the evaporation, suction was applied through the drying tube to aid in the distillation and drying.

The flask containing the dry ethyl magnesium bromide was cooled in ice water, and 75 cc. of dry *n*-propyl alcohol (distilled over CaO) were added in small portions through the top of the reflux condenser. The residue dissolved completely. The solution was then heated and refluxed for 10 minutes to decompose the last traces of the Grignard reagent. After cooling to 50°, 280 mg. of twice crystallized protoporphyrin dimethyl ester were added to the flask, arrangement being made to pass dry N₂ slowly into one arm of the flask. The flask was placed on a water bath and kept at 70–75° for 5 to 7 hours or until the protoporphyrin band at 630 mμ had disappeared. Higher temperatures or prolonged heating led to yellow decomposition products.

The material in the flask was now transferred, with the aid of a small amount of ether, to a Claisen distilling flask and the propyl alcohol distilled almost to dryness under diminished pressure in the presence of N₂. The dry material was now transferred with the aid of 750 cc. of water and 750 cc. of ether to a 2 liter separatory funnel. The solution was shaken to extract most of the Mg protoporphyrin ester into the ether. Then 100 cc. of a solution containing 10 gm. of ammonium acetate and 10 gm. of Na₂HPO₄ were added. A flocculent precipitate of MgNH₄PO₄ was produced. This was drawn off and the ether layer washed several times with water. A small amount of impurity went to the interphase and was drawn off. The ether solution was dried with anhydrous sodium sulfate and evaporated

to dryness under diminished pressure in the presence of N_2 . The dark red powdery residue was dissolved in some 30 cc. of wet ether and filtered. A small brownish residue with a band at 470 to 480 $m\mu$ collected on the filter and was discarded.

Even when pure, the Mg protoporphyrin ester is difficult to crystallize from solution, although crystals will be found to form on a glass slide under the microscope. Crystallization was accomplished in the following manner: To the concentrated ether solution, 3 cc. of xylene were added, and the solution further evaporated down to about 7 to 10 cc. Then 5 cc. of low boiling petroleum ether were added (b.p. 30–60°) and a crystalline precipitate rapidly formed. After cooling for several hours the precipitate was centrifuged, washed with low boiling petroleum ether by centrifuging, and then filtered off. The yield of this crystalline pinkish powder was 220 mg. or about 75 per cent of theory.

A portion of the pink powder was washed on the filter with anhydrous ether. The filtrate consisted of a colloidal solution; a slight residue remained on the filter paper. To the filtrate was added low boiling petroleum ether, and a precipitate of plates and highly twinned crystals resulted. This was centrifuged, washed with low boiling petroleum ether, and dried *in vacuo*. Analyses of this material showed the following percentage composition.

$C_{55}H_{50}O_4N_4Mg$.	Calculated.	C 70.5, H 5.89, N 9.15, Mg 3.98
	Found.	" 70.36, " 6.01, " 9.04, " 4.00

An aliquot of this crystalline material was dissolved in moist ether and the absorption spectrum of the red fluorescent pigment was determined (smooth curve, Fig. 3). The absorption bands are very sharp and high. The molar extinction per cm. of light path for a given wave-length is given by

$$E_{\text{mole per liter}}^{1 \text{ cm.}} = \log_{10} \frac{I_0}{I} \cdot \frac{1}{\text{cm.} \times \text{mole per liter}}$$

E at 419 $m\mu$ = 308,000, at 340 $m\mu$ = 20,550, at 551 $m\mu$ = 18,200, at 589 $m\mu$ = 18,200, and at 510 $m\mu$ = 2450.

A comparison of the position of the visible absorption spectra of various divalent metal mesoporphyrins which have been studied shows that the Mg compound has its bands furthest displaced toward the red end of the spectrum (4).

Preparation of Dipotassium Salt of Mg Protoporphyrin—30 mg. of the Mg protoporphyrin ester were treated in a 50 cc. centrifuge tube with 5 cc. of 30 per cent methyl alcoholic KOH for 15 minutes at 40°. Then 10 to 15 cc. of water were added, resulting in a flocculent precipitate. The precipitate was centrifuged down and the supernatant liquid discarded. The

precipitate was dissolved in 5 cc. of hot methanol and placed in the ice box. Crystals arose, consisting of rhomboid plates, often highly twinned, especially if rapidly formed (Fig. 5). If crystallization did not occur under these conditions, then crystallization could be induced by adding small portions of a solution made up by diluting the methyl alcoholic KOH 1:10 with water. The plates were dichroic, dark red and pale yellow. On the basis of $K_2C_{34}H_{30}O_4Mg$, calculated, N = 8.4 per cent; found, 8.2 per cent. The absorption spectrum of this compound was measured in 0.02 N KOH containing 50 per cent ethanol. The extinction values in this solution are lower than for the ester in the ether solution (Fig. 3).

Biological Activity - Hemophilus influenzae Turner requires heme or protoporphyrin for growth and for the reduction of nitrate to nitrite (4).

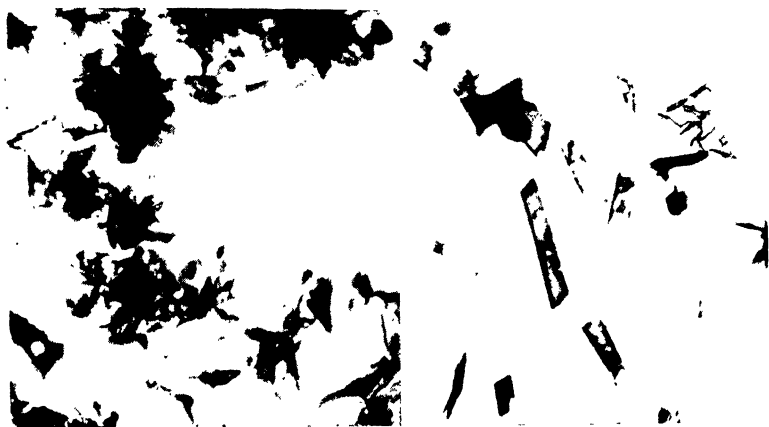


FIG. 5. Crystals of the dipotassium salt of magnesium protoporphyrin. Left-hand, formed rapidly from aqueous methanol, $\times 400$; right-hand, formed slowly from methanol, $\times 100$.

It was found that Mg protoporphyrin would support the growth of this organism at a concentration one-fifth of that of protoporphyrin. As with protoporphyrin, the nitrate reducing activity of organisms grown on Mg protoporphyrin was proportional to the growth of the organisms. A tentative explanation for the fact that the growth-promoting effect of magnesium protoporphyrin is greater than that of the protoporphyrin itself may be found in the fact that the magnesium porphyrin has a smaller tendency to form colloidal solutions and will therefore be more readily available to the cell than porphyrin itself. Once in the cell, it is probable that the magnesium is split out and then the iron inserted (4).

We desire to express our thanks to Dr. L. Michaelis for his constant stimulation and advice.

SUMMARY

From 100 cc. of cells of *Chorella* mutant 60, about 0.5 mg. of a pinkish fluorescent pigment was isolated. This was identified as Mg protoporphyrin in several ways. Its absorption spectrum agreed with the spectrum of synthetic magnesium protoporphyrin in position and relative heights of the bands. The metal was split out of the complex with acid and identified as Mg^{++} by the quinalizarin lake method. The porphyrin was identified as protoporphyrin by its HCl number and by its absorption spectrum.

Methods for the synthesis of magnesium protoporphyrin dimethyl ester and of the dipotassium salt of magnesium protoporphyrin are described.

The isolation of magnesium protoporphyrin suggests that, after the synthesis of protoporphyrin, insertion of magnesium is the next step in the biological synthesis of chlorophyll by *Chlorella*.

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THE SPECIFIC REFRACTIVE INCREMENT OF SOME PURIFIED PROTEINS

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A quantitative interpretation of the electrophoretic patterns of protein mixtures obtained with the aid of the Tiselius method is based, in part, on a knowledge of the specific refraction of the proteins to be analyzed. Since the patterns are recorded at 0.5° and since no precise refractive index measurements of proteins at this temperature are available, an investigation has been made of the specific refractive increment of some purified proteins. A differential prism method, developed in this Laboratory,¹ has been used in conjunction with the optical equipment of the electrophoresis apparatus. This permits data to be obtained under the same conditions as those encountered in the routine electrophoretic analysis of protein mixtures. The results of this investigation are presented in this report.

EXPERIMENTAL

The proteins that have been studied in this research are listed in Table I. The egg albumin and β -lactoglobulin were prepared in this Laboratory; the bovine serum albumin was obtained from the Armour Company, Chicago, and the samples of human serum albumin and γ -globulin were kindly supplied by the Department of Physical Chemistry, Harvard Medical School.² In the case of egg albumin the purification procedure, including three recrystallizations, was that used by Sørensen and Høyrup³ whereas β -lactoglobulin was prepared by a modification of Palmer's method⁴ and was recrystallized four times. The modification consists of the removal of casein by the addition of solid ammonium sulfate to 40% saturation. The ammonium sulfate concentration of the filtrate was then increased to 55% saturation, the precipitate discarded and the β -lactoglobulin prepared from the filtrate.

Except for the egg albumin, which was kept as a paste in a concentrated ammonium sulfate solution, all of the protein samples were stored at 2° as dry powders until used.

* Commonwealth Fund Fellow, 1945-1947.

(1) Longworth, *Ind. Eng. Chem. Anal. Ed.*, **18**, 219 (1946).

(2) The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

(3) Sørensen and Høyrup, *Compt. rend. lab. Carlsberg*, **12**, 1 (1915-1917).

(4) Bull and Currie, *THIS JOURNAL*, **68**, 742 (1946).

Salt-free solutions of the albumins were prepared by dialysis against distilled water. In the case of the protein solutions containing neutral or buffer salts, a sample was dissolved in the appropriate electrolyte solution and then dialyzed against this solution.

The concentrations of all of the protein solutions, except those dissolved in the sodium diethylbarbiturate buffers, have been determined from nitrogen analysis of weighed portions by the Pregl micro Kjeldahl method with the precautions recommended by Chibnall.⁵ The factors for conversion of these results to a dry weight basis have been determined as follows. In the case of the albumins, weighed portions of a salt-free isoelectric solution were used for both the nitrogen and dry weight determinations. The dried residue presumably consisted entirely of protein. In the

TABLE I
Nitrogen Content of the Purified Proteins

		Egg albumin	Bovine serum albumin	Human serum albumin	β -Lactoglobulin	Human γ -globulin
1	Method of desiccation	100° <i>in vacuo</i>	110° in air	110° in air	110° in air	110° in air
2	Percentage of nitrogen	15.72	16.05	15.95	15.53	15.9
3	Nitrogen factor	6.36	6.23*	6.27	6.44	6.29
4	References to nitrogen content	3,5	6	6,7	5,8	6,7

* The same value is obtained with water-dialyzed and electro-dialyzed samples.

case of γ -globulin, an isoelectric solution in aqueous sodium chloride was used and correction for the weight of the salt in the residue was made with the aid of the assumption that this was the same as in a mass of the dialysate equal to that desiccated. The factor for the β -lactoglobulin was obtained from a nitrogen determination on a desiccated sample of the solid protein, thus eliminating any uncertainty as to correction for salt in the residue.

Under the conditions of desiccation given in line 1 of Table I, it was found that the dried residue attained a constant weight in twenty-four hours. The resulting nitrogen factor, line 3, is in good agreement with the best values found in the literature, references to which are indicated in line 4.

The determination of the protein concentration in the presence of the barbiturate buffer will be described later in this paper.

All weight concentrations have been converted to a volume basis, *i.e.*, gram protein per 100 ml. solution, with the aid of the density data in the "International Critical

(5) Chibnall, Rees and Williams, *Biochem. J.*, **37**, 354 (1943).

(6) Brand, Kassel and Saidel, *J. Clin. Invest.*, **23**, 437 (1944).

(7) Cohn, Strong, Hughes, Jr., Mulford, Ashworth, Melin and Taylor, *THIS JOURNAL*, **68**, 459 (1946).

(8) Brand, Saidel, Goldwater, Kassel and Ryan, *ibid.*, **67**, 1524 (1945).

Tables"⁹ and in Svedberg and Pedersen's "The Ultracentrifuge."¹⁰ In estimating the density of a given solution the specific volumes of the components have been taken as additive. Moreover, all of the proteins of Table I have been assumed to have the specific volume, 0.741, of the "average protein" as given by Svedberg¹¹

RESULTS

Effect of Protein Concentration in Salt-Free Solutions.—As the results presented in Table II indicate, the specific refractive increment, $k = (n_{\text{solution}} - n_{\text{solvent}})/p$, of a protein is independent of its concentration, p , over a wide range

TABLE II

Effect of Protein Concentration on the Specific Refractive Increment in Salt-free Solutions

Protein	Protein concn., p , g./100 ml.	Specific refractive increment, $k \times 10^6$ at 0.5°
Egg albumin, pH 4.95	1.614	1874
	3.200	1877
	4.026	1878
	6.451	1877
		Av. 1876
Bovine serum albumin, pH 5.05	3.766	1906
	4.740	1902
	5.631	1906
	10.099	1897
		Av. 1901
Human serum albumin, pH 4.85	1.777	1886
	3.456	1887
	5.188	1888
	7.683	1887
		Av. 1887

of this variable, if the concentration is expressed on a volume scale, e.g., g./100 ml. solution. The results in Table II, and also those in Fig. 1 below, indicate that, although the specific refractive increment varies with the protein, this variation is small. These results confirm and extend those of Adair and Robinson¹² and others.

(9) "International Critical Tables," Vol. III, McGraw-Hill Book Co., Inc., New York, N. Y., 1928.

(10) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, 1940, p. 446, app. III.

(11) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, 1940, p. 445, app. II.

(12) Adair and Robinson, *Biochem. J.*, **24**, 993 (1930).

Effect of Temperature on the Specific Refractive Increment.—Since the differential prism method can readily be adapted for work over a range of temperature,

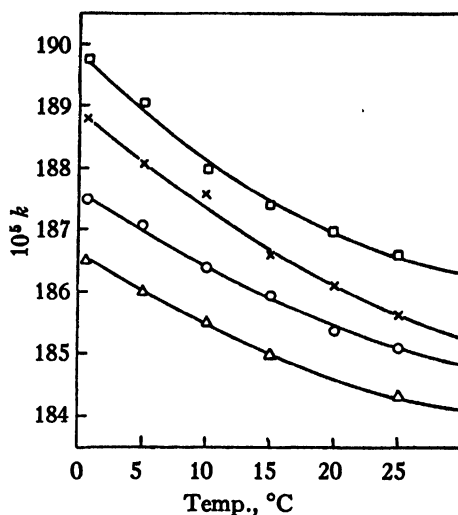


FIG. 1.—Effect of temperature on the specific refractive increment of proteins: □, bovine serum albumin; ×, human serum albumin; ○, egg albumin; Δ, β -lactoglobulin.

TABLE III

Comparison of the Effect of Temperature on the Specific Refractive Increment, k , of Proteins with its Effect on Other Substances

Substance	$k_0 \times 10^5$	$(k_0 - k_{25}) \times 10^5$
Sodium chloride		+17 ₁
Potassium chloride		+10 ₀
Glycine, alanine		+ 8 ₀
Arginine hydrochloride		+ 7 ₉
Glycylglycine		+ 8 ₀
Bovine serum albumin	190 ₁	+ 3 ₂
Human serum albumin	188 ₇	+ 3 ₃
Egg albumin	187 ₆	+ 2 ₅
β -Lactoglobulin	186 ₅	+ 2 ₃
Human γ -globulin	187 ₅	0

selected solutions have been studied at 0.5, 5, 10, 15, 20 and 25°. These results are presented in Fig. 1 for the three albumins and β -lactoglobulin. Here the specific refractive increment, k , is plotted as ordinate against the temperature as abscissa. Contrary to the general impression, the effect of temperature on the refractive increment is not negligible for most proteins although it is smaller than that observed in the case of salts. This is shown in Table III, which also

includes the results that have been obtained on some low molecular weight materials having electrical properties between those of salts and proteins. Of the substances investigated only γ -globulin had a negligible temperature coefficient.

The Specific Refraction at Different Wave Lengths.—Although most of the refractive index measurements have been made at $\lambda = 5780 \text{ \AA}$., the mean value of the mercury yellow doublet that is isolated with the Wratten filter number 22, a few solutions have also been studied at other wave lengths. In these

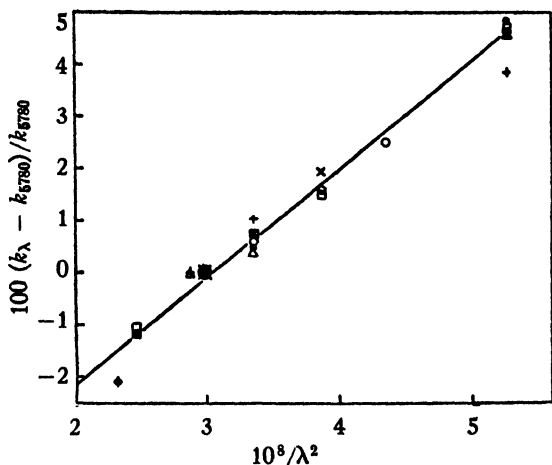


FIG. 2.—Effect of wave length on the specific refraction: □, bovine serum albumin; ×, human serum albumin; O, egg albumin; Δ, β -lactoglobulin; +, horse serum albumin; ●, horse serum globulin.

measurements a cadmium-mercury lamp of the H4 type is used without a filter, the cell is filled with a fairly concentrated protein solution and, with the aid of the cylindrical lens attachment, the spectral lines are observed directly in the focus of the schlieren camera. With the yellow line as reference the displacements of other lines are measured and the corresponding values of $k_\lambda - k_{5780}$ computed. In Fig. 2, values of $(k_\lambda - k_{5780})/k_{5780}$ are plotted as ordinate against the reciprocal of the square of the wave length as abscissa. As will be seen in the figure, all of the points, including those for horse serum albumin and globulin, and β -lactoglobulin taken from the work of Pedersen and Anderson,^{13, 14} can be adequately represented by a single straight line. Although the specific refraction varies somewhat with the nature of the protein it thus appears that the dispersion is essentially the same for all the proteins that have been studied. Since the slope of the line in Fig. 2 is 0.1946 the relation $k_\lambda =$

(13) Pedersen, *Biochem. J.*, **30**, 961 (1936).

(14) McFarlane, *ibid.*, **29**, 407 (1935).

$k_{5780} (0.940 + 2.00 \times 10^6/\lambda^2)$, where λ is in ångström units, may be used to obtain the specific refraction at any (visible) wave length.

The Specific Refraction of Sodium Proteinates.—The protein solutions employed in an electrophoretic analysis are usually prepared by dialysis against

TABLE IV

Effect of Charge on the Specific Refractive Increment of Crystalline Egg Albumin and Bovine Serum Albumin

1 Moles NaOH $\times 10^4$ per 1 g. protein	2 pH	3 p	4 Δn	5 $A' \times 10^4$	6 a
Egg albumin					
00.00	4.95	6.451	0.01210 ₆	1877	—
13.03	5.70	5.951	.01123 ₄	1888	45
19.28	6.20	5.739	.01086 ₈	1894	47
25.38	7.26	5.545	.01052 ₃	1898	44
32.67	8.7	5.334	.01018 ₇	1908	53
42.3	10.1	5.128	.00977	1904	36
44.6	10.5	5.025	.00959 ₃	1909	34
					Mean 44
Bovine serum albumin					
00.00	5.02	4.740	0.00901 ₅	1902	—
19.59	7.22	4.340	.00832 ₉	1919	46
34.44	8.32	4.077	.00788 ₅	1934	49
53.68	10.10	3.782	.00735	1944	41
64.75	10.58	3.606	.00703 ₆	1951	40
72.97	10.72	3.525	.00697 ₈	1979	56
					Mean 46

an appropriate buffer solution. The proteins are then present as charged particles in a solution of buffer ions whose composition is given, as a first approximation, by the Donnan equations. If the pH of the solution is above the isoelectric pH of the protein and if, as is usually the case, sodium buffer salts are employed, the protein is present as a sodium proteinate whose specific refraction differs from that of the isoelectric protein. Moreover, in addition to the acquisition of a net charge the protein may also bind some of the buffer salt. In order to distinguish between the effect of the charge and of bound salt it is essential to determine the refraction of sodium proteinate in the absence of buffer salts for comparison with the values obtained in their presence. Consequently the specific refractions of salt-free isoelectric protein solutions, to which small amounts of sodium hydroxide have been added, were determined with the results given in Table IV.

In this table the quantity of alkali added is given in column 1 and the resulting pH of the solution in the next column. The protein concentration, column 3, is the value for the isoelectric solution after correction for the dilution due to the added alkali. If this is divided into the observed refractive increment, column 4, a specific refraction, k' , column 5, is obtained that differs from the

TABLE V

Effect of Sodium Chloride on the Specific Refractive Increment of Crystalline Egg Albumin, Bovine Serum Albumin and Human Serum Albumin

Soln. no.	Protein	Solution prepared by	pH	ρ	$k \times 10^4$
1	Egg albumin	Dialysis vs. H_2O	5.31	3.154	1869
2		Dialysis vs. 0.1 M NaCl	5.47	3.555	1874
3		Electrodialysis	4.74	3.187	1869
4		Dialysis vs. H_2O	4.95	3.199	1876
5		Dialysis vs. 0.5 M NaCl	5.38	3.604	1869
6	Bovine serum albumin	Dialysis vs. H_2O	5.05	4.740	1902
7		Dialysis vs. 0.1 M NaCl	5.35	5.219	1932
8		Dialysis vs. 0.5 M NaCl	5.31	4.634	1948
9		Dialysis vs. 0.1 M NaCl		3.495	1938
10		Dialysis of soln. No. 9 vs. H_2O		4.539	1919
11		Electrodialysis	5.25	2.223	1923
12		Electrodialysis + dialysis vs. 0.1 M NaCl	5.37	4.103	1943
13		Electrodialysis		4.891	1921
14		Electrodialysis + dialysis vs. 0.1 M NaCl		5.438	1941
15		Dialysis of soln. No. 14 vs. H_2O		6.424	1920
16	Human serum albumin	Dialysis vs. H_2O	4.85	3.424	1887
17		Dialysis vs. 0.5 M NaCl	5.26	2.262	1918

specific refraction k , of the isoelectric protein by an amount that is proportional to the net charge, e , of the protein, *i. e.*

$$k' = k(1 + \alpha e)$$

Here the net charge is given by the values of column 1 since all of the added alkali reacts at the pH values studied. The computed values of the proportionality factor, α , column 6, are approximately constant and are essentially the same for the two proteins studied.

Refraction Measurements in Solutions in Sodium Chloride.—In view of the possibility that ions other than the hydrogen ion may be involved in the dissociation equilibria of the proteins, refraction measurements on these materials in solutions of the neutral salt, sodium chloride, have been made as an additional prerequisite to the study of proteins in buffer solutions. The results presented in Table V, where the specific refraction is taken as the difference,

per unit concentration of protein, between the refractive indices of the equilibrated solutions, illustrate the two types of behavior that have been encountered. Thus the specific refraction of egg albumin is essentially independent of the concentration of the sodium chloride against which it has been dialyzed, whereas in the case of the bovine and human serum albumin small but significant changes occur. This is consistent with the findings of Scatchard and his associates,¹⁵ that these two materials bind some sodium chloride.

An additional feature of the bovine serum albumin used in this research also emerges from the data of Table V. Thus the k -values of 1923 and 1921×10^{-6} for the electrodialed solutions Nos. 11 and 13 differ significantly from that of 1902×10^{-6} for the water-dialyzed solution No. 6. If, however, water dialysis is used to remove the salt from solutions that have been equilibrated with aqueous sodium chloride, solutions Nos. 10 and 15, the values obtained, 1919 and 1920×10^{-6} , then agree well with those for the solutions prepared by electrodialysis. The origin of these effects is obscure but, together with the observed constancy of the nitrogen factor, suggests the presence in the bovine serum albumin of a volatile, poorly refracting contaminant that is removed by electro- and saline dialysis but not by the ordinary water dialysis. Alcohol or decanol as the contaminant might satisfy these requirements.

Refraction Measurements in Buffer Solutions.—In the following interpretation of the refraction measurements in buffer solutions the assumptions have been made (a) that the refractions due to the various components are additive, (b) that the net charge on the protein may be obtained from the titration curve and (c) that the buffer electrolyte concentrations are given by the first term in the expansion of the Donnan equation. With the aid of these assumptions, together with the measured refractive increment, $\Delta n_{\text{obsd.}}$, of the protein solution and that, $\Delta n'_{\text{obsd.}}$, of the buffer solution against which it has been equilibrated, the specific refraction of the sodium proteinate has been computed as will be described in connection with Table VI. In that table, the concentrations, in equivalents per liter, of the buffer electrolytes in the dialysate, the pH of the protein solution in equilibrium therewith and the protein are given in the first four lines. The protein concentrations in line 5 marked with an asterisk were obtained by mixing weighed amounts of the buffer solution and the protein. Corrections were made not only for moisture content of the protein but also, by rapid weighing of the Cellophane bag and its contents before and after dialysis, for the rather large volume changes that occur during this process. In the case of the concentrations marked with a dagger a weighed sample of the equilibrated protein solution was dialyzed salt-free, transferred quantitatively to a volumetric flask and nitrogen determinations were made on weighed portions of this salt-free solution. It will be noted that the two methods of analysis lead to closely agreeing results for the specific refraction in the one instance

(15) Scatchard, Batchelder and Brown, *THIS JOURNAL*, **68**, 2320 (1946).

where a comparison is possible, *i.e.*, columns III and IV of Table VI. In the phosphate buffers the protein concentration was obtained in the conventional manner since these buffer salts do not interfere with the nitrogen determination.

The sources of the values for the net charge, line 6 of Table VI, are given as footnotes whereas the equivalent concentration of the sodium proteinate, line 7, is simply $C_{\text{NaP}} = -10pe$. In the case of the diethylbarbiturate buffers the

TABLE VI

The Specific Refractions of Proteins in Buffer Solutions

BSA = bovine serum albumin. HSA = human serum albumin. EA = egg albumin.
LG = lactoglobulin.

	I	II	III	IV	V	VI	VII	VIII	IX	X
	R = diethylbarbiturate ⁻						R = NaHPO ₄ ⁻			
1 $C'(\text{NaR})$	0.025	0.050	0.1	0.1	0.1	0.1	0.128	0.134	0.134	0.128
2 $C'(\text{HR})$	0.005	0.01	0.02	0.02	0.02	0.02	0.008	0.008	0.008	0.008
3 ρH	8.56	8.59	8.60	8.60	8.60	8.60	7.67	7.71	7.74	7.68
4 Protein	BSA	BSA	BSA	BSA	EA	LG	BSA	HSA	EA	LG
5 $p - \text{g./100 ml. soln.}$	2.010 ^a	2.056 ^a	1.9193 ^a	5.024 [†]	1.8115 [†]	2.695 [†]	3.4977	2.9943	2.3565	2.8324
6 $-e$	0.00038 ^a	0.00038 ^a	0.00038 ^a	0.00038 ^a	0.00032 ^a	0.00051 ^b	0.00027 ^a	0.00027 ^c	0.00027 ^a	0.00043 ^a
7 $C(\text{NaP}) = -10pe$.00764	.00781	.00729	0.1909	.00580	.01374	.00944	.00808	.00648	.01218
8 $C(\text{NaR})$.02118	.0461	.0963	.0905	.0971	.0931	.1220	.1286	.1297	.1202
9 $C(\text{HR})$.005	.01	.02	.02	.02	.02	.0078	.0078	.0079	.0078
10 $\Delta n(\text{obsd.} - \text{corr.})$.004986	.006323	.008291	.014179	.008087	.009800	.008665	.007767	.006520	.007297
11 $\Delta n(\text{NaR} + \text{HR})$.001004	.002160	.004486	.004251	.004518	.004356	.001966	.002066	.002084	.001939
12 $\Delta n(\text{NaP})$.003982	.004163	.003805	.009928	.003569	.005444	.006699	.005701	.004436	.005358
13 $k(\text{NaP})$.001981	.002025	.001982	.001976	.001970	.002020	.001915	.001904	.001882	.001892
14 $k(\text{NaP}) \mu \rightarrow 0$.00193 ₅	.00193 ₅	.00193 ₅	.00193 ₅	.00190 ₂	.00190 ₉	.00192 ₃	.00191	.00189 ₉	.00190 ₁

^a Interpolated from data of Table IV. ^b From Cannan, Palmer and Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

^c Cohn, Strong, Hughes and Blanchard, see Edsall, *Annals N. Y. Acad. Sci.*, **47**, 223 (1946).

electrolyte concentrations in the protein solutions, lines 8 and 9, are given by the relations¹⁶

$$C_{\text{NaR}} = C'_{\text{NaR}} - \frac{1}{2}C_{\text{NaP}} \text{ and } C_{\text{HR}} = C'_{\text{HR}}$$

whereas for the phosphate buffers¹⁷

$$C_{\text{NaR}} = C'_{\text{NaR}} (1 - 5C_{\text{NaP}}) \text{ and } C_{\text{HR}} = C'_{\text{HR}} (1 - 2.5C_{\text{NaP}})$$

Owing to small variations of the equivalent refraction with concentration and also to the difficulties of preparing some of the buffer salts for weighing, deviations of as much as 1×10^{-6} in the measured refractive increments of the buffer solutions from those computed by means of the relation

$$\Delta n' = K_{\text{NaR}}C'_{\text{NaR}} + K_{\text{HR}}C'_{\text{HR}}$$

have been observed. Here K is the equivalent refraction and, for the computations of Table VI, has been assigned the following values: sodium diethyl-

(16) Longworth, *J. Phys. and Coll. Chem.*, **51**, 171 (1947).

(17) Svensson, *Arkiv. Kemi, Mineral o. Geol.*, **22A**, No. 10, 27 (1946).

barbiturate, 0.04055; diethylbarbituric acid, 0.02905; Na_2HPO_4 , 0.01502; and NaH_2PO_4 , 0.0172. Correction for the deviations has been made by adding to the observed increment for the protein solution the small difference between the computed and observed increments for the buffer solution. The corrected values of the refractive index increment for the protein solutions are given in line 10 of Table VI.

The contribution of the buffer electrolytes to the increment of the protein solution is given by the relation

$$\Delta n(\text{NaR} + \text{HR}) = K_{\text{NaR}}C_{\text{NaR}} + K_{\text{HR}}C_{\text{HR}}$$

line 11 of Table VI, and the difference

$$\Delta n(\text{NaP}) = \Delta n - \Delta n(\text{NaR} + \text{HR})$$

line 12, divided by the protein concentration is the specific refraction of the sodium proteinate, line 13. For comparison, the values of k for the sodium proteinates in the absence of salt have been interpolated from the data of Table IV for the pH values of Table VI and are given in the last line of that table. It will be noted that the specific increment in the phosphate buffer is essentially the same as in the absence of salt whereas in the presence of the diethylbarbiturate it is significantly greater. Although several explanations for this observation could be advanced an attractive one is that this large organic ion is bound by the protein.

Comparison with Previous Work.—As a test of the validity of the differential prism method used in this research two of the salt solutions studied by Hölemann and his associates^{18, 19} have been prepared and their refractive increments determined over a sufficient range of temperature and wave length to permit a direct comparison with their results. At 25° and $\lambda = 5876 \text{ \AA.}$, the increments for 1.6692 molal sodium chloride and 1.2983 molal potassium chloride are 0.015385 and 0.011906, respectively, and are in agreement with the values of 0.01538 and 0.01190 reported by Hölemann. Additional evidence that the precision of our refractive index measurements is about $\pm 1 \times 10^{-6}$ is afforded by the following fact. At 0.5° and $\lambda = 5780$, the refractions of the solutions of potassium chloride recently used in diffusion studies²⁰ follow, with an average deviation of $\pm 7 \times 10^{-6}$, the simple relation, $\Delta n/C = 0.011405 - 0.00100\sqrt{C}$, over the concentration range studied, i.e., 0.1 to 1.0 normal.

In the case of the specific refractions of proteins, however, the precision of the results is limited by the uncertainty in the protein concentration. As Armstrong, Budka, Morrison and Hasson have shown,²¹ the presence of lipid in the protein renders particularly difficult the concentration determination. In the

(18) Hölemann and Kohner, *Z. physik. Chem.*, **B13**, 338 (1931).

(19) Shibata and Hölemann, *ibid.*, **13**, 347 (1931).

(20) Longworth, *THIS JOURNAL*, **69**, 2510 (1947).

(21) Armstrong, Budka, Morrison and Hasson, *ibid.*, **69**, 1747 (1947).

present research this source of error has been reduced by restricting our studies to proteins that contain minimal amounts of lipid and by controlling the nitrogen determinations with dry weight measurements. In spite of these precautions, however, in unfavorable cases the uncertainty in the protein concentration may amount to as much as one per cent. In some instances this is sufficient to mask the variation of the specific refraction from one protein to another or between different preparations of the same protein.

It is of interest that at 20° our values of the specific refraction of human albumin, 1862×10^{-6} , Fig. 1, and γ -globulin, 1875×10^{-6} , agree well with those, 186×10^{-5} and 188×10^{-5} , reported by Armstrong and associates. In the case of β -lactoglobulin, Pedersen reports¹³ 1809×10^{-6} and 1812×10^{-6} as the specific refraction, at 20° and $\lambda = 5799 \text{ \AA}$., of two different preparations. In these measurements he used nitrogen factors of 6.61 and 6.55, respectively, that were obtained from nitrogen and dry weight determinations on aliquots of a solution of the protein in 0.5 molar sodium chloride. When analyzed similarly, our preparation of this protein gave a factor of 6.62, in essential agreement with Pedersen's values. However, when analyzed, as described earlier in this paper, in such a manner as to eliminate the uncertainty concerning the amount of salt in the dried residue, the nitrogen factor is 6.44. Correction of Pedersen's refraction data to this new factor gives $10^6 k = 1858$ and 1843, in satisfactory agreement with our value of 1846 at 20°.

The authors are glad to acknowledge their indebtedness to D. A. MacInnes of these Laboratories for his interest in this research and for suggestions in the preparation of this paper.

SUMMARY

With the aid of a hollow, prismatic cell and the optical equipment of the Tiselius electrophoresis apparatus, the refractive index increments of solutions of some purified proteins have been measured as a function of the protein concentration, the temperature, and the wave length of the incident light. The changes in the specific refractive increment that occur on titration of the protein with alkali, in the presence of neutral salts and after equilibration with buffers have also been determined. Such data are necessary for a quantitative interpretation of the electrophoretic patterns of proteins.

THE EFFECT OF GROWTH OR RETROGRESSION OF A TRANS-PLANTABLE LYMPHOSARCOMA OF THE RAT ON THE LYMPHOID ORGANS AND THE ADRENALS OF THE HOSTS

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The material used in this investigation was a rapidly growing, transplantable lymphosarcoma of the rat, which has been propagated in this laboratory since 1940 (2). The cells of this tumor inoculated intraperitoneally give rise to typical leukemia, but if inoculated into the subcutaneous tissue of the groin, a localized sarcoma develops that may metastasize to regional lymph nodes but rarely progress to generalized disease. At present, the tumor takes in from 60 to 70 per cent of the inoculated animals and, when progressive, grows rapidly to a very large size, causing death in about 20 days. In the routine autopsies on the inoculated rats, considerable variations were noted in the size of the lymphoid organs. An analysis of these observations follows.

OBSERVATIONS

The weights of the thymus, pooled cervical and axillary lymph nodes, spleen, and adrenals were taken on these organs from 159 rats made up of the following groups: (a) 53 rats with actively growing tumors, (b) 71 rats with tumors in various stages of retrogression, and (c) 35 normal rats of the same strain, age, and average weight, which had been kept under the same laboratory conditions as the inoculated animals.

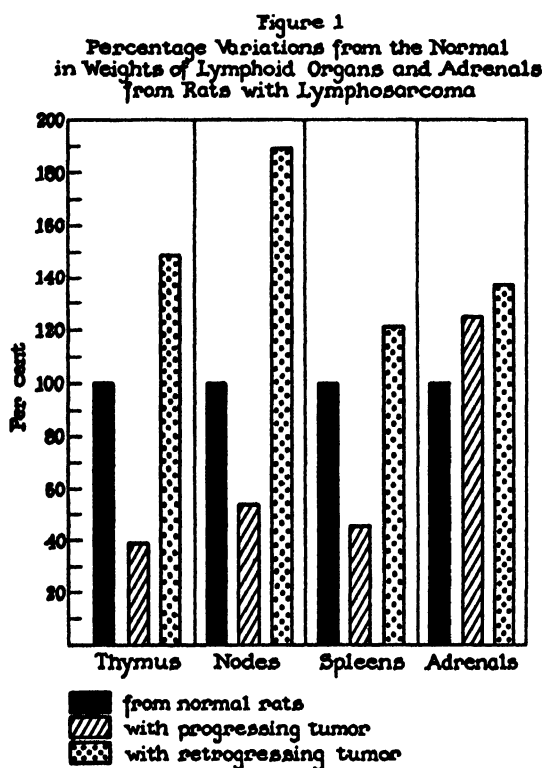
The average weights of the organs from the three groups are given in Table I and the percentage variation from the normal is shown graphically in Fig. 1. It will be noted that the most pronounced difference is between the weight of the lymphoid organs of the rats with progressing tumors and those with retrogressing tumors. The average thymus weight of the group with progressing tumors, in the 31 of the 53 rats which showed detectable thymus tissue, was only 38 per cent of the average weight of the normal thymus. The average weight of this organ from animals with retrogressing tumors was increased by 49 per cent over the normal. The same general trend was observed in the lymph nodes, except that the average increase in weight of the nodes in rats with retrogressing tumors was more pronounced, being increased by 89 per cent over the normal weight. The spleen showed a comparable loss of weight in rats with growing tumors but in animals with retrogressing tumors the increase in the size of the spleen over the normal was not so pronounced as was that shown

by the thymus and nodes. The organs from one group of animals are illustrated in Fig. 2.

TABLE I

	No. of Rats	Rats	Thymus	Average Weights Nodes	Spleen	Adrenals
		gm.	mgm.	mgm.	mgm.	mgm.
Normal	35	126	224.0	177.2	943.3	31.9
With progressive tumors	53	124	85.5*	96.9	422.6	40.4
With retrogressive tumors	71	123	335.1	334.9	1140.1	43.65

* No thymus in 41.5%



In light of the previously reported indications (3, 4) that the adrenals and cortical hormones have an influence on the animal's resistance to this transplanted disease, the variations in average weights of these organs has an added interest. The glands from both the groups, one with growing tumors and the other with retrogressing tumors, showed a definite hypertrophy. In the former









































<u>Progressing Tumors</u>			<u>Regressing Tumors</u>		
Tumor size	Spleen	Thymus Mgm.	Spleen	Thymus	Mgm.
6.8 x 4.8		- 0			386.8
6.7 x 4.5		- 0			333.8
6.6 x 4.0		- 0			319.6
6.3 x 4.1		- 0			
6.2 x 4.2		- 0			303.8
5.9 x 4.0		- 0			
5.8 x 3.9		- 0			297.4
5.6 x 3.8		- 0			354.4
5.0 x 3.8		 85.8			
4.3 x 3.0		 199.2			300.2
4.4 x 3.1		 189.6			341.8
3.6 x 2.5		 158.6			295.8
					370.6
					287.8
					370.8

FIG 2

the increase in weight of the adrenals was 26.4 per cent over the normal, while in the latter the increase was 36.5 per cent. The average weights of adrenals from 16 rats in which the tumors had completely regressed were 55 per cent over the normal. Further analyses of the weights of adrenals from rats with retro-

gressing tumors show that those from animals killed 15 days after inoculation averaged 37.3 mgm. (22 rats) while those from animals killed from 25 to 29 days after averaged 49 mgm. (26 rats), an increase over the normal of 53 per cent.

DISCUSSION

The marked loss in weight of the principal lymphoid organs in animals with a rapidly growing lymphosarcoma may be the result of an uneven competition between the malignant and normal lymphoid cells for some essential nutritional factor. The definite hypertrophy of these organs, particularly the thymus and lymph nodes in rats with retrogressing lymphoid tumors, is not immediately explainable. In a previous study of the lymphoid organs in mice resistant to inoculated carcinoma, it was noted that there was a hyperactivity in the lymphoid tissues manifested by increase in mitoses, size of germinal centers, and numbers of cells in the circulation, but there was no consistent increase in weight of the lymphoid organs (1).

Excessive amounts of adrenal cortical hormone may reduce the size of lymphoid organs to as great an extent as that which occurs in the animals with rapidly growing lymphosarcoma. Even though such animals have some hypertrophy of the adrenals it is doubtful whether this is sufficient to account for the extent of the change. An even greater hypertrophy of the gland in rats with retrogressing tumors is not sufficient to prevent the great increase in weight of thymus and nodes found in such animals. In fact, the extent of this increase in thymus and nodes is similar to that observed to follow the complete removal of the adrenals.

SUMMARY

The weight of thymus, lymph nodes, and spleen from rats with actively growing transplanted lymphosarcoma are much reduced from the normal weights of these organs, the thymus by 62 per cent, nodes by 56 per cent, and the spleen by 55 per cent. Conversely, these organs are definitely increased in weight in rats bearing tumors in the process of retrogression. The lymph nodes show an increase of 89 per cent, the thymus 49 per cent and the spleen 21 per cent over the normal. The adrenals of rats with progressing tumors were found to average 25 per cent above the normal weights of these organs, while those from animals with retrogressing tumors had an increase of over 30 per cent. In the latter group the degree of hypertrophy was more marked when complete retrogression of the tumors had taken place (53 per cent).

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A PARTICULATE BODY ASSOCIATED WITH EPITHELIAL CELLS CULTURED FROM MAMMARY CARCINOMAS OF MICE OF A MILK-FACTOR STRAIN*

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PLATES 5 TO 7

(Received for publication, March 5, 1948)

The occurrence of mammary tumors in mice has been shown to be influenced by a transmissible agent, by an inherited tendency to develop breast cancer, and by hormonal stimulation of the gland tissue. Since the initial report of the existence of an extrachromosomal factor (1) and the demonstration by Bittner of its presence in the milk (2), many investigators have sought a definition of its mode of action, its transmission, and its character. Their results have shown the agent to have many of the characteristics of a virus (3-5). In order to define the nature of the agent more precisely, attempts have been made to study it with the electron microscope. At the time of this writing, we are aware of only two previous accounts of such efforts. Graff *et al.* (6) have examined the ultracentrifugate of milk of high-cancer and low-cancer strains of mice. In a brief note they report that the high cancer strain milk contains a "heavy particle" which "has virus-like dimensions." Passey *et al.* (7) made water extracts of desiccated normal and malignant breast tissue from mice of high- and low-cancer strains. In micrographs of material from high-cancer strains they found a particulate component about 200 Å in diameter which, they report, was not present in extracts of tissue from low-cancer strains. Both of these observations, the latter more than the former, are subject to the criticisms that the agent may be greatly altered by the preparation procedures and may be easily confused in microscopy with particulate elements present in the cytoplasm of all cells. A study of the cells themselves would be less subject to these criticisms and might, moreover, give some information on the mode of reproduction of the agent and its relation to the tissue cells.

Earlier reports from this laboratory have disclosed the suitability of cultured cells for electron microscopy (8) and have demonstrated that the method can be used not only for the study of new cytological detail (9) but can be applied as well to the identification of viruses in or on cells (10) and to the study of the special cytological features of malignant cells (11). It seemed worth while to

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† Fellow of the Finney-Howell Research Foundation.

use similar techniques in an investigation of the cells from mammary tumors of the mouse. The following is a report of the initial efforts.

Materials and Methods

The cells for study were grown from explants of both spontaneous and transplanted tumors. In all, four spontaneous and two transplanted tumors have been used. The former originated in female mice of the high-tumor strain C₃H, a strain in which the milk factor is known to be operative; the latter were fifth generation transplants of a spontaneous tumor¹ that also; arose in a C₃H mouse. All tumors were typical adenocarcinomas of the mammary gland that is to say they had the character of the growths known to be determined by the milk factor.

Cultures were prepared on formvar-coated slide inserts in roller flasks by methods described already (8). The explants were placed in shallow clots made up of equal parts of nutrient and chick plasma diluted 1:4 with Tyrode's solution. The nutrient was composed of 5 parts Tyrode's, 3 parts human cord serum, and 2 parts chick embryo extract. Each set of cultures was made with tumor tissue from a single animal, and successive sets were separated by intervals of 2 weeks or more. Different lots of cord serum, plasma, and embryo extract were used on each set. When, after a few days of culturing, small sheets of epithelial cells were obtained, the explants were removed, the remaining cells were washed briefly in a slow stream of Tyrode's (pH 7.4) and then placed in the vapor of osmium tetroxide for fixation. After periods over OsO₄, varying from 2 to 24 hours, the cells were mounted on screens and dried for electron microscope examination. All the micrographs were taken with an RCA (type E.M.U.) instrument.²

OBSERVATIONS

The culture conditions of the experiments seemed adequate, since within 2 days of culturing most of the explants showed surrounding sheets of epithelial cells. These continued to spread during the 3rd and 4th days, at the end of which time they were usually fixed. Occasionally the cells of an epithelial sheet were observed to cytolize rather suddenly to be replaced later by a new growth.

A typical epithelial sheet is shown in Fig. 1. It is apparent that many of the cells are thinly spread and hence satisfactory for electron microscopy. Only small portions of a cell can be micrographed in any one exposure, so most of the illustrations include only a minute area approximating that outlined (A) in Fig. 1.

The electron microscope examination of preparations of the epithelial sheets had not gone far before it was observed that numbers of a small, apparently spherical particle were associated with some of the cells (Fig. 4). The characteristic density and morphology of these particles set them off from the normal cytoplasmic components (Figs. 5 and 7). In some cells they lay scattered in

¹ Designated as Law 916.

² The microscope was generously loaned to the project by Dr. R. M. Taylor, Director of the laboratories of the International Health Division of The Rockefeller Foundation.

small numbers (Figs. 4 and 5) while in others they literally packed the cell (Fig. 3).

Thus far in the material examined, the particles have been found associated only with epithelial cells or fragments of these cells. They can be seen in all parts of the cell and are not especially abundant nearer any particular component of it. Occasionally they appear to be within the endoplasmic strands and mitochondria (Figs. 4 and 5), and a few micrographs have shown them in the area of the nucleus (Fig. 3). It is difficult, however, to be certain whether they are actually within these structures or merely superimposed, the latter relationship seeming more probable in the case of the nucleus. Figs. 4 and 5 depict the more ordinary random association, and micrographs such as these show that the particles are generally situated in the ectoplasmic substance of the cytoplasm which, in these well extended cells, forms a thin layer between the membranes.

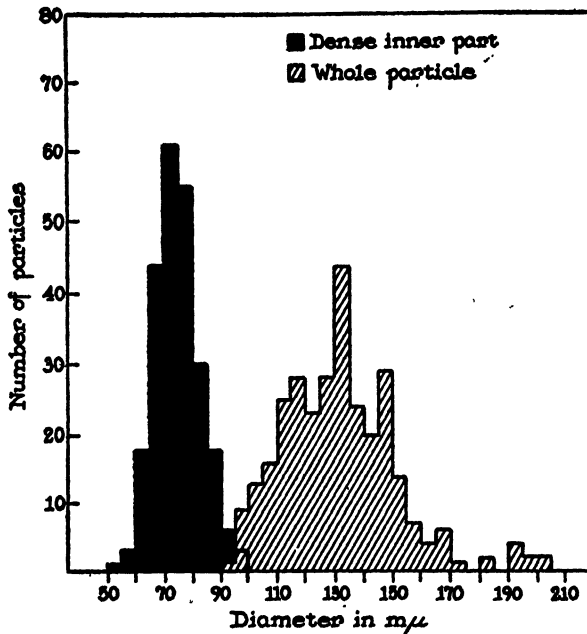
Small patches of cell membrane, sometimes with great numbers of particles on them, have been frequently found (Fig. 7). Evidently, while being washed prior to fixation, the cell proper was removed by the stream of Tyrode's and only portions of the membrane next to the formvar remained. The presence of the particles on such fragments indicates that in the intact cell they were located just within the cell membrane. Preparations of this sort are particularly valuable for good microscopy, since there is no overlying membrane and but little cytoplasm around the particles to scatter electrons and thereby reduce the definition. Similar fragments of cell membrane and other portions of the cell, probably products of natural cytolysis, have also been observed with associated particles, sometimes great numbers of them. Fig. 2 shows the particles definitely located on the cell surface. The significance of this observation is lessened, however, by the possibility that the cell may have ruptured in this region, releasing the particles shown.

The particles themselves are remarkably constant in character (Figs. 2 to 7). Their form appears to be spherical. Close examination of the micrographs (Figs. 4 and 6) reveals that most of them have a dark or dense center that is quite sharply defined and set off from a less dense, capsule-like periphery. This double structure is probably accentuated by OsO_4 fixation and may be consequent on the more osmiophilic properties of the central substance. In electron micrographs of gold-shadowed preparations it is possible in some cases (Fig. 5) to note that the central dense portion protrudes above a flattened border. This suggests that in desiccation the outer zone dries down more than the central part of the particle.

The diameter of the central, dense core is fairly constant from particle to particle and averages approximately $75 \mu\mu$ (Text-fig. 1). The over-all dimension is more variable and averages about $135 \mu\mu$. Measurements on shadowed material indicate that the height of the dried particle is approximately one-half

the width. This is taken to mean that some flattening of the spherical form results from drying. The greater variation shown by the outside diameters (Text-fig. 1) is doubtless due in part to the fact that they are often poorly defined and not so accurately measurable. There is a suggestion in these findings as well that this portion of the particle is readily distorted, as if it were less rigid or viscous than the core.

The particles may occur singly, in pairs, or in clumps of all sizes. Some



TEXT-FIG. 1. Histogram to show size distribution of particles. It was constructed from 238 measurements of the diameter of the inside dense portion of the particle and 303 measurements of the over-all diameter. The values of these two average approximately 75 mμ and 130 mμ respectively.

groupings are of special interest. In Fig. 6 (A), for example, one can see at least two rosette arrangements formed by a single row of granules surrounding a central, unusually large particle. Clusters of four bodies, two large and two small, as in Fig. 7, are fairly common. It should be mentioned also that some of the unusually large particles look as if compound in structure; *i.e.*, composed of two, four, or more parts within a single capsule. The larger of such "encapsulated" clumps may have diameters almost double that of the average particle. Whether such special groupings of particles are stages in the reproduction of the typical form will have to be determined by further study. For studies of this type, the cultured material is especially favorable because

the particles are left relatively undisturbed and still associated with the tissue cell in which they are presumably multiplying.

Table I lists the origins of the material examined and shows the observed occurrence of the particles. It will be noted that in the preparations obtained from three out of six experiments none of the special sort described has been

TABLE I

Experiment	Date	Source of tumor	Age of tumor	Mouse strain	Age of cultures when used	Total no. screens	No. with particles	Quantity of particles
	1947		whs.		days			
I	Apr. 18	Law 916* Transplanted 5th generation 3 × 3 × 2 cm.	6	C ₃ H†	5-7	19	0	
II	May 14	Law 916* Transplanted 5th generation 3 × 3 × 2.5 cm.	11	"	7	12	1	Large numbers associated with the cells of only one small epithelial sheet
III	Aug. 29	Spontaneous 1 × 1 × 1 cm.	2	C ₃ H† Lactating ♀	4	24	21	Isolated to abundant
IV	Nov. 24	Spontaneous 1 × 0.8 × 0.8 cm.	2	C ₃ H†	2-4	38	0	
V	Dec. 4	Spontaneous 1.5 × 1 × 1 cm.	6	"	3-8	14	0	
VI	Dec. 12	Spontaneous 1 × 0.7 × 0.7 cm.	5	"	3-6	46	25	Isolated to abundant

* Law 916, transplantable adenocarcinoma that arose spontaneously in a high-tumor strain C₃H mouse.

† Mammary tumor incidence approximately 90 per cent.

found. This does not prove that none was there, it means only that none was encountered during a moderately extensive examination of the screens. In the single preparation of transplanted tumor cells in which the particles were noted they were found only over a small area of the screen, in connection with a few cells. Screens from Experiment III (Table I), on the other hand, showed cells carrying great numbers of particles. Most of the illustrations were taken from this material. Particles were likewise more generally present

and abundant in the preparations of Experiment VI. Thus in these very limited studies the cells from spontaneous tumors derived from high tumor incidence C₃H mice have more commonly shown the granules than have those from transplanted tumors of similar derivation propagated in such mice.

DISCUSSION

These studies have disclosed the frequent presence in mouse mammary tumor cells of a particulate body, having a fairly uniform size, density, and morphology. Though similar in size, and possibly in other respects, to some normally occurring cytoplasmic granules (11), these particles are sufficiently different from the latter to give the impression of being special entities. Their uniform morphology, their association in closely packed clumps, as well as their irregular occurrence in tumor cells are especially significant as features distinguishing them from normal components of the cell. These same features make it seem probable that they are of extraneous origin and that they may be a virus.

The double structure shown by the particles cannot be explained as merely the image of a spherical body of uniform density. The latter, in an electron micrograph, would also show a relatively dark central portion, but its density should grade off gradually toward the periphery instead of suddenly altering to give a much lighter outer zone. Though evidence is lacking for any accurate interpretation of this finding, it does suggest the existence of a nuclear-like body surrounded by an envelope or capsule of different character. Electron micrographs of the viruses of equine encephalomyelitis (12), influenza A and B (13, 14), and vaccinia (15) have shown them to possess a similar complexity.

The variation in size recorded in the histogram (Text-fig. 1) is doubtless the reflection of a number of factors. Inaccuracy of measurement due to poor marginal definition will account for some of it; but the readily discernible differences in the diameter of the particles pictured make it clear that much of the variation is significant. As mentioned above, extremely large bodies have been seen that apparently consist of several small granules within a single "capsule." Possibly such large units are actually clusters of several small entities that have had their origin from a single particle. Conceivably, these small entities might enlarge to form separate particles of average size arranged around a primary one as in the rosettes shown in Fig. 6.

The most obvious, and for the moment, important question arising from these observations is whether or not the particles represent the milk agent of the mammary tumors. At present, there appears to be no simple and direct way of determining this; it will be necessary, instead, to gather evidence tediously through a comparison of tumor cells obtained from growths presumably carrying the milk agent with cells from the mammary tumors of agent-free animals. Such a study is in progress. Meanwhile, it may not be amiss

to consider the facts which favor identification of the particles with the milk agent. Numerous preparations of other cells from different species of animals, cultured in the same media as these mammary tumor cells, have not shown any particles of like appearance. It follows that they cannot have derived from the media. Secondly, in this connection, it seems significant that the particles are associated only with the epithelial cells of the cultures. Fibrocytes observed in the same preparations appear not to carry them. Other cell types from the mouse and from other species have been examined over the last 2 or 3 years, and no granules of precisely the same character have been encountered.

In view of these considerations and the fact that the cells were derived from tumors that arose in a high-tumor strain, it seems reasonable to assume tentatively that the particles are in fact the milk agent. In making this assumption we are aware of the possibility that the cells showing the particles may have come from tumors carrying an intercurrent virus. In this connection attention should be called to the tremendous difference in size between the particles reported upon here (1350 Å) and those observed by Passey *et al.* (7) in electron micrographs of material from mouse mammary tumors. In water extracts of tumors presumably carrying the agent these investigators found "approximately spherical particles about 200 Å in diameter" whereas in extracts of agent-free tumors nothing of the sort was observed. It is difficult to believe that this difference could be entirely a product of different preparation techniques. But further speculation may best be postponed until such time as Passey and his collaborators have tested the activity of their extracts and until the present studies of cultured cells have been extended to control material.

The electron microscope as a means for detecting intracellular inclusions is far from infallible, and unless the particles now under consideration are present in significant numbers or in clumps, they could be easily overlooked. This might account for their apparent absence in preparations made from some of the tumors, notably the two transplanted tumors studied. Yet it is possible that their scarcity in preparations from these latter represents a genuine scarcity. If so, the observation would tie in with some observations of Barnum, Ball, and Bittner (16) that the agent in transplanted tumors does not give as high a titre as in the spontaneous tumors.

It will be noted in Table I that in one set of preparations studied the particles appeared in the cells in tremendous numbers. The mouse from which these cells came was lactating at the time, and possibly this condition was attended by a multiplication of the agent. Conceivably also, the cells, if harvested an hour or two earlier, might have shown far fewer particles, whereas a little later they might have been destroyed.

Finally, it should be mentioned that a significant gain from these studies and our examination of normal cells, has been the experience needed for the

recognition of virus-like bodies associated with tissue cells. As one acquires the ability to discriminate the unusual or abnormal, the prospect develops that yet more pathological material can be examined with profit.

SUMMARY

Epithelial cells from spontaneous and transplanted mammary adenocarcinomas developing in high-tumor strain C₃H mice have been grown *in vitro* and studied with the electron microscope. In preparations from three out of six tumors, an unusual particulate body has been found associated with the cells. The particles appear to have a spherical shape and a double structure consisting of a dense center and less dense outer zone. The diameter of the central dense portion is fairly uniform from particle to particle, averaging approximately 75 m μ ; whereas the outside, whole particle diameter is more variable and averages about 130 m μ . From the micrographs it would appear that these peculiar virus-like bodies are situated chiefly in the ectoplasmic portion of the cell. They may occur singly, in pairs, or in clumps of varying sizes. Cells containing great numbers of the particles show signs of degeneration, and cell fragments are frequently encountered with many particles on them.

So far, the particles have been found only in association with the epithelial cells of the cultures. They are apparently not derived from the culture media. All in all the findings are consonant with the view that the particles represent the milk agent. Further evidence for or against this assumption is being sought from a study of cells from normal tissue and tumors demonstrated to be agent-free.

We are greatly indebted to Dr. Lloyd Law for his stimulating interest and the requisite material. It is a pleasure to acknowledge as well the technical assistance of Margaret Carr and Julia Hine.

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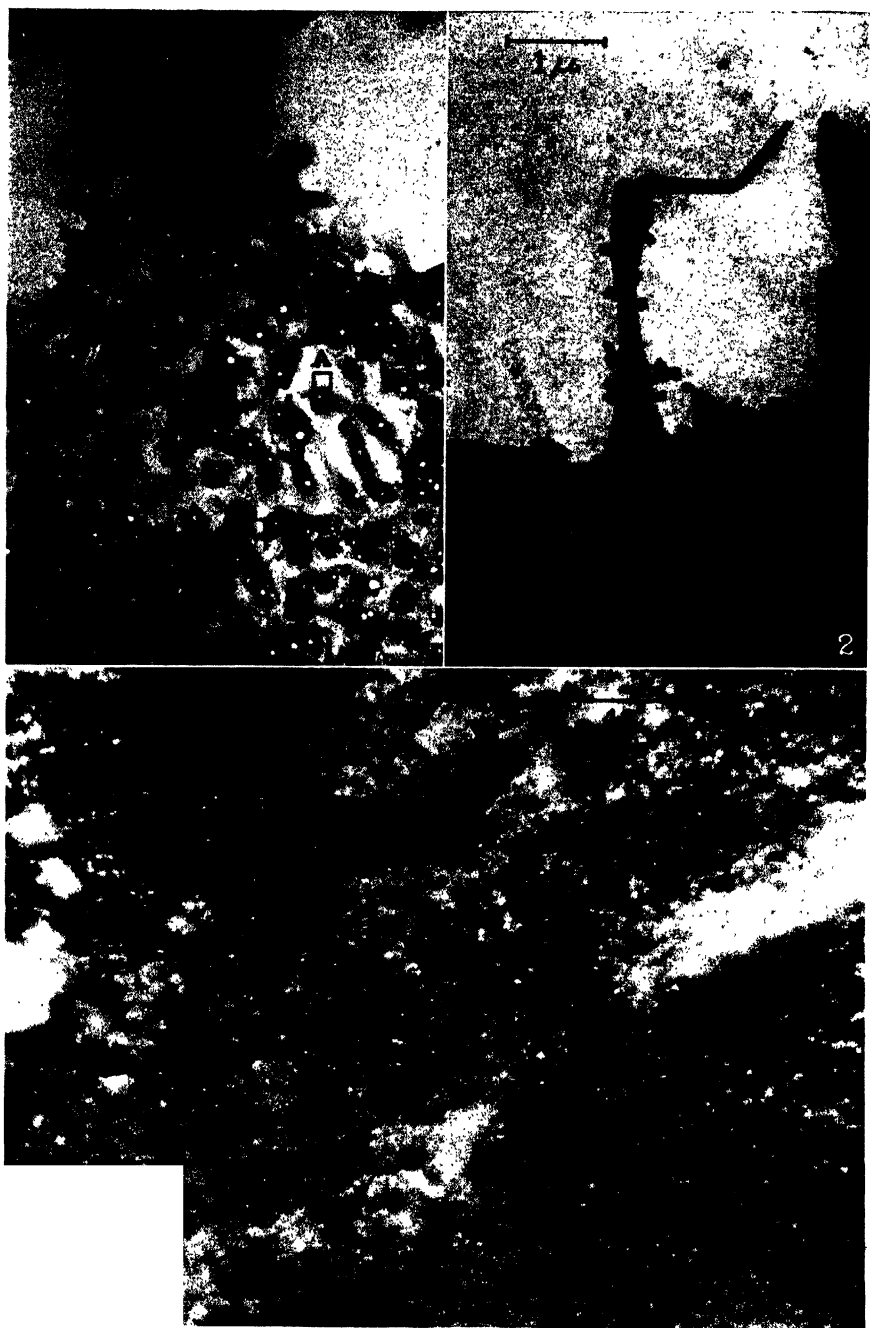
EXPLANATION OF PLATES

PLATE 5

FIG. 1. Photomicrograph of a typical sheet of epithelial cells grown from a spontaneous tumor explant. It illustrates the type of material that is suitable for electron microscopy. Small areas of approximately the size outlined (A) can be included in a single micrograph and the other figures are electron micrographs of such cell portions. The vacuoles are without significance in the present study. Taken from a 2-day-old culture, fixed in saline-formalin and stained with hematoxylin and eosin. $\times 200$.

FIG. 2. Electron micrograph of a portion of a cell margin showing particles attached to the edge of the cell and to pseudopodia projecting from the cell. At the bottom of the micrograph is the cell body. It is possible that the particles on the outside have been released from the cell through disruption of the membrane. It is to be noted that outside as well as inside the cell the particles possess a double structure which may be taken to indicate that the "capsule" is an integral part of the particle. Preparation made from 4-day-old culture of a spontaneous mammary tumor explant, fixed over vapor of OsO_4 24 hours. $\times 14,000$.

FIG. 3. Electron micrograph of a portion of a thick cell selected because it showed better than any other cell encountered in the preparation how gross the infection of particles may become. The large dark area outlined in white and shaped like a quadrant of a circular disc, which can be seen at the lower right-hand corner of the picture is part of the nucleus of the cell. The many particles which can be seen in this area may or may not be within the nucleus. Probably, as indicated by the definition of the image, they are between the cell and the nuclear membranes. Preparation from a 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO_4 . Shadowed with gold at an angle of 10° . $\times 12,500$.



(Porter and Thompson: Virus-like bodies in mammary carcinoma cells)

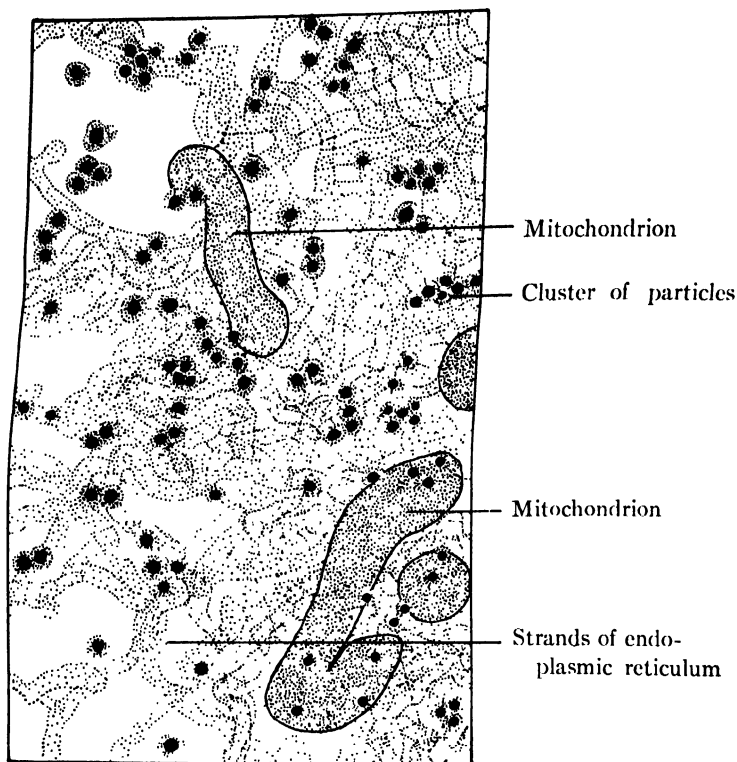
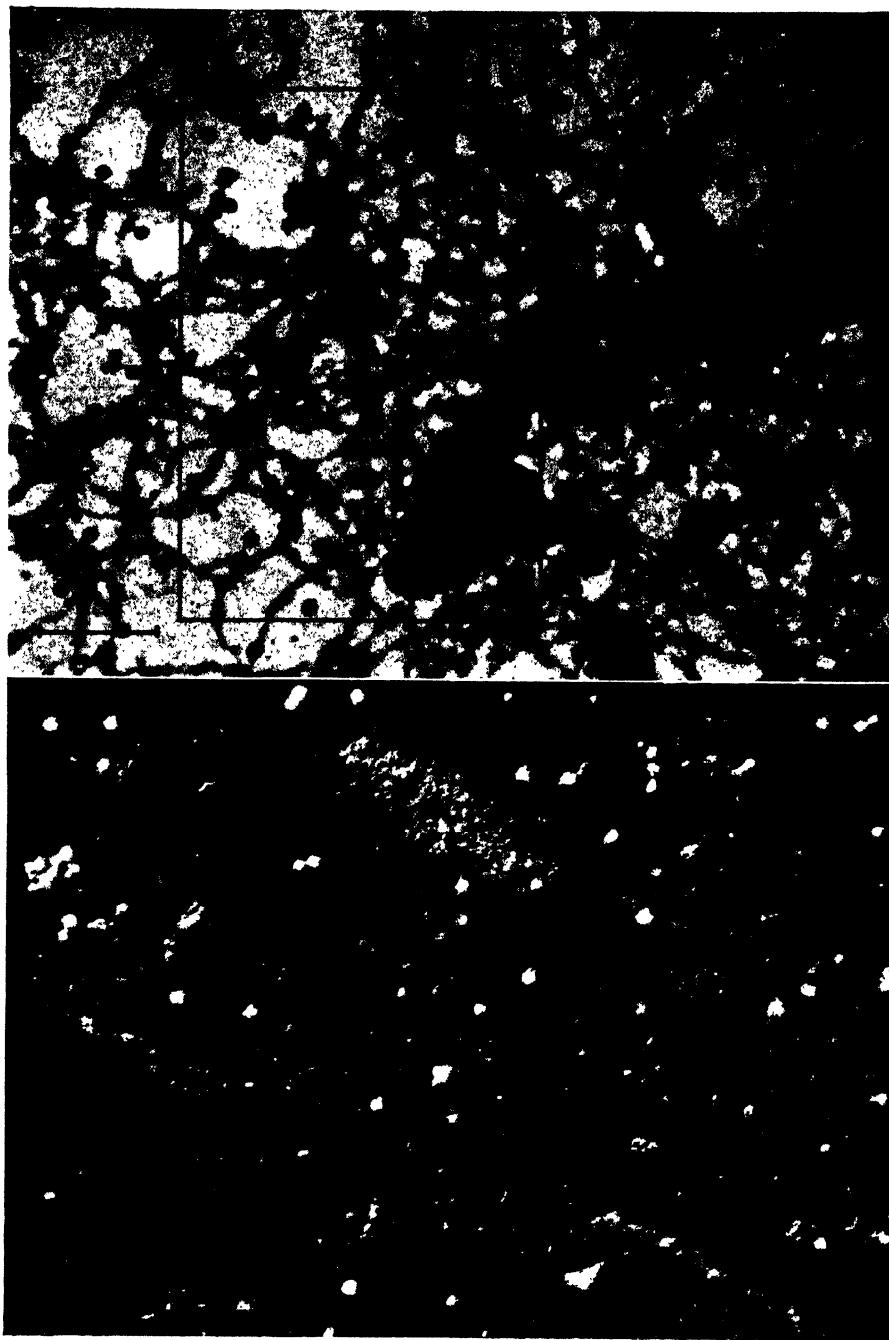


FIG. 4 *a*

PLATE 6

FIGS. 4 and 4 *a*. Electron micrograph of a small area of an epithelial tumor cell from a spontaneous C₃H mammary gland carcinoma growing in culture. Fig. 4 *a* is a descriptive diagram of a portion of it. The particles occur singly or in clumps of various sizes and are scattered about without apparent association with any particular cytoplasmic component. The relatively large mitochondria lie amidst strands of the endoplasmic reticulum. The upper cell membrane seems to be intact. Apparently the particles are situated in the ectoplasmic substance between the cell membranes. Some of them can be seen to show a central density and a peripheral "capsule." Preparation from a 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄. $\times 17,000$.

FIG. 5. Electron micrograph of a cell similar to that shown in Fig. 4, demonstrating by means of gold shadowing the three dimensional form of the dry particle contained in a cell. It can be seen that they have not dried down as much as the surrounding material and hence project up through the cell membrane, casting gold-free shadows. The mitochondria and strands of the endoplasm have flattened in drying. Some of the particles appear to be within the strands of endoplasm but may actually be superimposed. Preparation made from 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄. Shadowed with gold at an angle of 10°. $\times 17,000$.



(Porter and Thompson: Virus-like bodies in mammary carcinoma cells)

PLATE 7

FIG. 6. Electron micrograph showing several clumps of virus-like particles. These were found in a cell of a small epithelial sheet from an explant of a transplanted tumor. It can be noted that the individual particle has a dense center and a less-dense periphery, and that there is a distinct variation in size. Curious rosette arrangements of some of the particles are indicated by (A). Preparation from a 7-days old culture, fixed over vapor of OsO_4 20 hours. $\times 21,500$.

FIGS. 7 and 7a. Electron micrograph of a cell membrane with particles attached and descriptive diagram of a portion of it. During the preparation of the cells for fixation, the superficial part of the cell was washed away, leaving merely a small portion of the membrane next the formvar. One margin of this fragment runs across the upper left-hand corner of the picture, and its other edge across the lower right-hand corner. The finely granular background is the inside surface of the cell membrane. Presumably its granular character is due to structural units of the membrane or to adsorbed macromolecules. The line with the deep shadow to the right is the real edge of the cell and the full thickness of the latter can be seen extending upward. In this triangular lighter area both membranes are present. The virus-like particles are the larger white bodies. They stand up approximately spherical and cast long shadows. The numerous smaller and flatter biscuit-shaped bodies are vesicles of the cytoplasmic endoplasm such as are commonly seen in normal cells.

It can be noted that the shadowed particles show considerable variation in size. The indicated arrangement of two small and two large particles has been observed in other micrographs.

Preparation made from a 4-day-old culture, fixed 18 hours over vapor of OsO_4 . Shadowed with gold at an angle of 10° . $\times 20,000$.

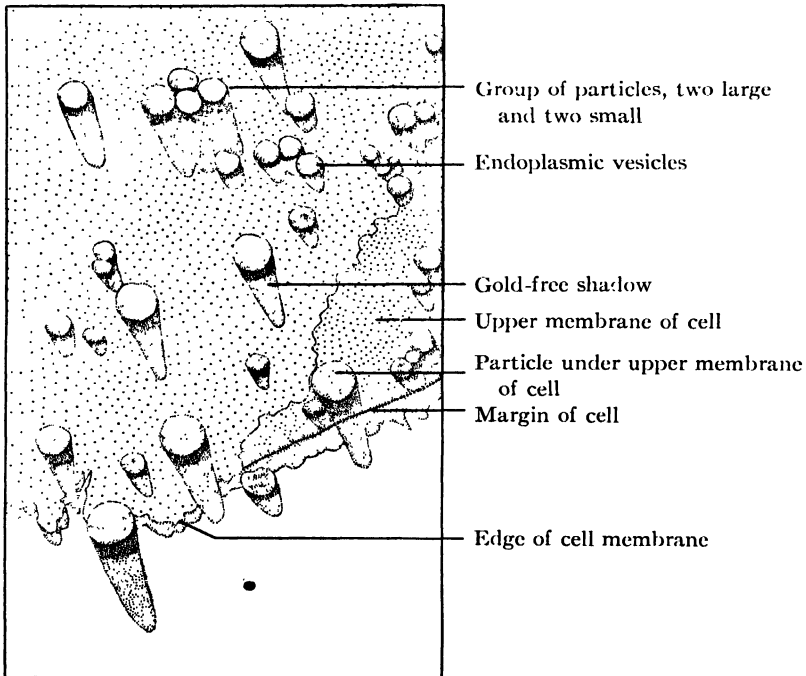
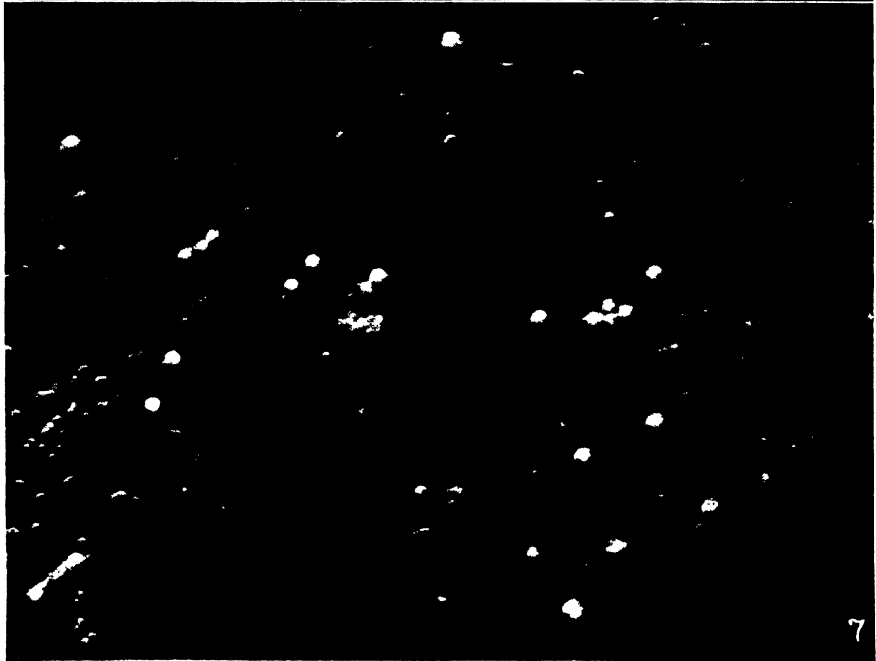
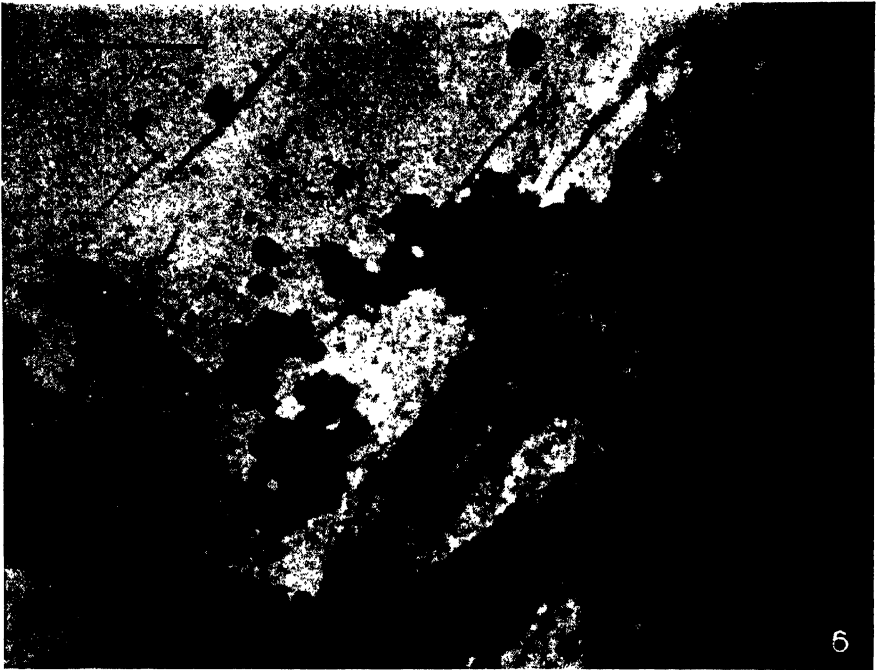


FIG. 7a



(Porter and Thompson: Virus-like bodies in mammary carcinoma cells)

PURIFICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE BY COUNTER-CURRENT DISTRIBUTION*

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The isolation in the pure state of the coenzyme diphosphopyridine nucleotide (DPN) has constituted a difficult problem in chemical fractionation for a number of years. At the present time, it is possible by means of relatively simple procedures to obtain crude preparations containing 40 to 60 per cent DPN from yeast in good yield (1-3). Further purification of these crude preparations (4-7) usually involves precipitation of DPN with cuprous chloride, subsequent removal of acid impurities as insoluble salts of heavy metals, adsorption of DPN on columns of Al_2O_3 , and finally fractional precipitation with alcohol. The procedure is tedious and is not easily reproducible with respect to the purity or yield of the final product.

The formidable nature of this method of purification has, in fact, led to the wide-spread use of crude DPN in enzyme studies and related investigations. Although in most instances the known specificity of the DPN-linked dehydrogenases leaves little doubt that DPN itself takes part in the enzyme reactions, the question continually arises as to whether the impurities, of which the chemical nature is largely unknown, include interfering compounds. It is obvious that a reproducible and simple method for obtaining pure DPN in good yield would be desirable.

In the present report a new method, based on the counter-current distribution principle developed by Craig (8), is described for the fractionation of crude preparations of DPN (purity approximately 60 per cent). Recent applications of this technique of fractionation to other complex mixtures (9-12) have amply demonstrated that it is a powerful tool in the separation and characterization of organic compounds. By utilizing a two-phase system consisting principally of phenol and water, it has been possible with relatively few transfers to isolate DPN of high purity (at least 96 to 98 per cent pure) in yields of 70 to 80 per cent.

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EXPERIMENTAL

Since the procedure of counter-current distribution depends upon the use of a two-phase system, some difficulty would be anticipated in attempts to apply the method to compounds characterized by high solubility in water and low solubility in the common organic solvents. DPN is, of course, an excellent example of this type of compound. Even when distributed in systems that contained large amounts of water in the organic solvent phase, such as 1-butanol-water or 2-butanol-water, over 99 per cent of the DPN was found in the aqueous phase ($K < 0.01$). Phenol and certain of its derivatives (e.g. *m*-cresol), however, constituted a group of organic solvents allowing for more favorable conditions for the counter-current distribution of DPN. In the system water-phenol at 6°, for example, approximately 90 per cent of the DPN was found in the organic phase ($K = 0.12$). A preliminary counter-current distribution of fifteen transfers was made at 6° with 50 mg. of crude DPN with the system 2 volumes of water-1 volume of phenol. The results of this distribution showed that most of the impurities in the preparation possessed a much higher distribution coefficient than did DPN itself. Furthermore, DPN did not undergo an appreciable degree of transformation during the procedure. With these data at hand, it was possible to make a more detailed analysis of crude preparations of DPN by means of the counter-current distribution technique.

Materials and Methods—The crude DPN fractionated in the present experiments consisted of several different lots of cozymase obtained from the Schwarz Laboratories¹ and containing approximately 60 per cent DPN.

Reagent grade crystalline phenol (Merck) was redistilled under reduced pressure and stored in the liquid form by the addition of 10 per cent water. All other solvents used were also redistilled in glass.

The counter-current distribution machine available for the present experiments was equipped with a glass plate at each end,² making it possible to observe directly the separation of the liquid phases. This improvement was particularly useful in the purification of DPN because the water-phenol system separated rather slowly and had a tendency to emulsify in the presence of some of the impurities in cozymase. The settling of most systems is indicated simply by transmission of light through the tubes. In the case of water-phenol, however, the layers were frequently cloudy, and a determination of separation was made by tilting the machine to the horizontal position for observation of the solvent interface by reflected light.

The DPN content of the cozymase and purified preparations obtained by

¹ The authors are indebted to the Schwarz Laboratories, Inc., for a generous supply of cozymase.

² Craig, L. C., and Post, O., unpublished work.

distribution was estimated according to the method of Warburg and Christian (13) as modified at the Schwarz Laboratories.³ A 3 to 5 mg. sample (weighed to 0.01 mg.) was dissolved in 2.00 ml. of a freshly prepared solution containing 0.20 per cent $\text{Na}_2\text{S}_2\text{O}_4$ and 1.0 per cent NaHCO_3 . The mixture was placed in a boiling water bath for exactly 1 minute, immediately chilled in an ice bath, and diluted to an appropriate volume with a buffer containing 1.0 per cent NaHCO_3 and 1.0 per cent Na_2CO_3 . The solution was oxygenated for 5 minutes and its optical density measured at $340\text{ m}\mu$ in the Beckman spectrophotometer. This procedure was found to be superior to the usual method of reducing DPN over a period of several hours at room temperature (13) in that it gave more reproducible extinction coefficients.

The extinction coefficient at $340\text{ m}\mu$ afforded a precise measurement of the relative increase in purity and the yield of DPN obtained in the fractionation procedure. Although the extinction coefficient of pure DPN reduced in solution with hydrosulfite has not been definitely established, for purposes of convenience a value of 8.5 sq. cm. per mg., according to LePage (3), was employed to estimate the DPN content of the cozymase and the samples of purified DPN.

Analysis of Crude DPN by Means of Counter-Current Distribution—Fig. 1 shows the results of a twenty-four transfer distribution of 202 mg. of cozymase. The components of the system for distribution were equilibrated in the following proportions before the experiment: 200 ml. of water, 100 mg. of KCl, 90 ml. of phenol, and 10 ml. of ether. Each tube of the machine contained 12 ml. of the upper (aqueous) layer and 7.8 ml. of the lower (phenol-ether) layer. The pH of the aqueous layer was approximately 5. The distribution was carried out in a cold room at 6° in order to minimize hydrolysis of DPN.

Potassium chloride was added because it had been previously noted that the addition of a small quantity of a strong electrolyte to the water-phenol system prevented to a considerable extent the formation of emulsions. It was necessary to use an electrolyte which would not be an objectionable contaminant in preparations of purified DPN and which was soluble in ethanol, since the subsequent isolation of DPN involved precipitation from aqueous solution with ethanol. KCl satisfied these requirements reasonably well. Ether was added to raise the distribution coefficient of DPN in the water-phenol system. As can be seen in Fig. 1, the addition of ether gave a higher distribution coefficient ($K = 0.59$) which was more favorable for fractionation.

Upon completion of the distribution, the mixture in each tube of the machine was transferred to a glass-stoppered test-tube and extracted three

³ Gutcho, S., and Stewart, E. D., unpublished work.

times with 15 ml. of ether. The initial ether extraction resulted in the transfer to the aqueous phase of the material dissolved in the phenol and in the removal of the phenol from the system. Two additional extractions were carried out in order to insure complete removal of phenol. There remained twenty-five aqueous solutions containing the components of the

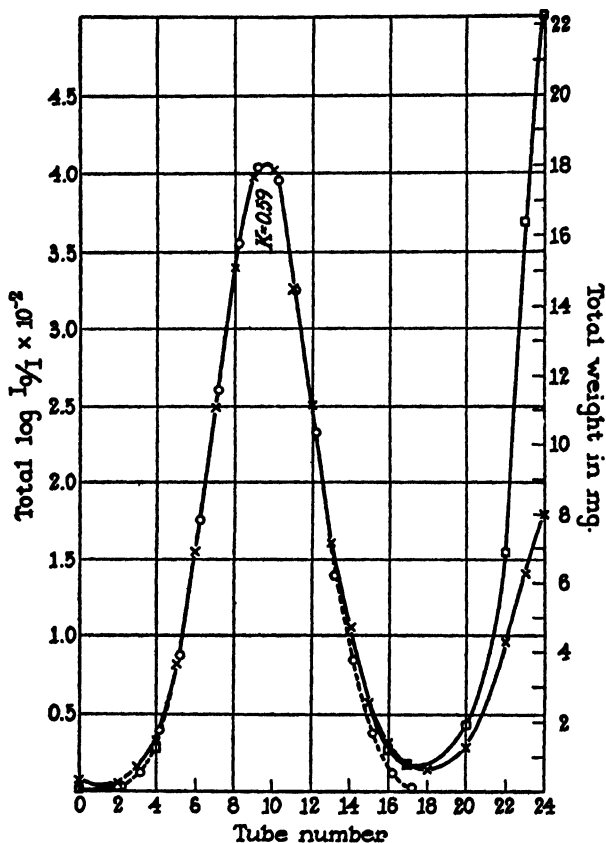


FIG. 1. Counter-current distribution of 202 mg. of cozymase in the system water-phenol-ether. X, total optical density at 260 $m\mu$; O, theoretical distribution of a single substance possessing a distribution coefficient of 0.59; □, total weight in mg. after evaporation to dryness.

cozymase and the KCl. These solutions were analyzed by determining their optical density at 260 $m\mu$ and the results plotted as shown in Fig. 1. The central band, which represented DPN, coincided very closely with the theoretical distribution of a single substance possessing a distribution coefficient of 0.59. There was, however, a slight deviation beginning at Tube

13 and extending to Tube 17, indicating the presence of another substance capable of absorbing light at $260\text{ m}\mu$. On the basis of absorption measurements, practically all of the remaining impurities in the cozymase were in Tubes 18 to 24. It should be mentioned that the impurities in Tubes 23 and 24 were troublesome during the distribution because they tended to cause emulsification of the system, making it necessary to wait 6 to 8 minutes between transfers.

Since by the analytical method only substances absorbing light at $260\text{ m}\mu$ could be detected, additional data were necessary before the central band could be satisfactorily interpreted. Accordingly, a number of samples from tubes on both sides of the band were evaporated to dryness and the weight of the residue determined. After correction for the amount of KCl present, the weights were plotted in Fig. 1 on a scale approximating the weight of material in the tubes of the central band. The latter values were estimated from the extinction coefficient at $260\text{ m}\mu$ of the DPN isolated from Tubes 6 to 12, inclusive. A consideration of the amount of material present in Tubes 2, 4, 16, and 17 led to the conclusion that the central band, on a weight basis, could have been no broader than the band determined by absorption measurements and shown in Fig. 1. This finding confirmed the results of a preliminary experiment involving fifteen transfers, in which it was found that the total weight of material present in each tube of the band representing DPN corresponded exactly to the optical density at $260\text{ m}\mu$. It was concluded, therefore, that Tubes 4 to 12 of the central band contained, except for KCl, either a single substance or a mixture of substances possessing identical distribution coefficients in the system.

Tubes 18 to 24 contained impurities amounting to 27 per cent of the weight of the original cozymase. The absorption curve of the material in these tubes showed a pronounced maximum at $260\text{ m}\mu$, typical of a compound or compounds containing adenine. On reduction with hydro-sulfite, however, the absorption of these impurities did not increase at $340\text{ m}\mu$, a finding that showed the absence of compounds containing quaternary pyridinium nitrogen. The large deviation between the weights and the absorption measurements in Tubes 22 to 24 of Fig. 1 demonstrated the presence of impurities other than those absorbing at $260\text{ m}\mu$.

Although very little residue remained after evaporation to dryness of the aqueous solutions obtained from Tubes 0 and 2 (Fig. 1), it should be mentioned that a yellow precipitate formed at the interface on ether extraction of the contents of Tubes 0, 1, and 2. This precipitate, which represented an impurity possessing a low distribution coefficient, was lost during the ether extraction, and its weight could not be estimated.

Isolation of DPN—Immediately after completion of the absorption analysis, the aqueous solutions obtained from Tubes 6 to 12 were combined,

filtered with suction through an inverted filter, frozen in a dry ice-acetone mixture, and lyophilized. The resulting solid material was dissolved in 4 ml. of water, and a white flocculent precipitate was obtained on addition of 40 ml. of cold absolute ethanol. The precipitate was recovered by centrifugation, washed with absolute ethanol and ether, and dried over P_2O_5 *in vacuo*. A white solid weighing 92 mg. was obtained. In an attempt to decrease the possibility of contamination with KCl, the nucleotide was redissolved in 3 ml. of water and reprecipitated with ethanol. The second precipitation presented some difficulties, however, because the compound formed an extremely fine precipitate consisting of particles of fairly uniform size (diameter about 0.4μ) that did not coalesce over a period of 18 hours at 0° . When recovered by centrifuging at $2400 \times g$ for 1 hour and dried over P_2O_5 *in vacuo*, the reprecipitated material weighed 82 mg. After reduction with hydrosulfite, its extinction coefficient at $340 m\mu$ was 8.13 sq. cm. per mg., indicating a purity of 96 per cent. The starting material contained approximately 62 per cent DPN ($E_{240} = 5.3$ sq. cm. per mg.).

Fractionation of Cozymase in System, Water-Phenol-Chloroform—The experiment shown in Fig. 1 demonstrated that most of the impurities in the cozymase possessed a much higher distribution coefficient than did DPN in the system, water-phenol-ether, and could therefore be effectively removed by a few transfers. It was evident, however, that twenty-four transfers were required to eliminate impurities possessing a low distribution coefficient. That such impurities were likely to occur in crude preparations of DPN was apparent from the detection of solid material in Tubes 0, 1, and 2, which appeared on extraction with ether but was lost on removal of the ether. It was therefore desirable to devise a system in which the distribution coefficient of DPN was approximately 1. By this means it would be possible to separate with relatively few transfers impurities possessing both high and low distribution coefficients. The addition of more than 10 per cent ether to the phenol layer of the water-phenol system, in order to obtain a distribution coefficient higher than 0.59, was not feasible, however, because the density of the phenol-ether phase then approached too closely that of the aqueous phase, and the system did not separate readily. Preliminary experiments in which increasing amounts of chloroform were added to the water-phenol system showed that a distribution coefficient of approximately 1 for DPN could be obtained when the three components were used in the following proportions: 15 ml. of water, 7 ml. of phenol, and 3 ml. of chloroform. When KCl was added to the aqueous phase at a concentration of 0.10 mg. per ml. of water, the system separated very rapidly.

A twenty-four transfer distribution of 600 mg. of crude DPN was then carried out in the water-phenol-chloroform system, each tube of the

machine containing 12 ml. of the aqueous layer and 7.8 ml. of the organic solvent layer. The two phases were obtained by equilibrating before the experiment the components of the system in the following proportions: 200 ml. of water, 20 mg. of KCl, 70 ml. of phenol, and 30 ml. of chloroform. The preparation of DPN available for this experiment had been partially purified by a previous fifteen transfer distribution in the system, water-phenol-ether. The extinction coefficient of the starting material at 340 $m\mu$ after reduction with hydrosulfite was 7.4 sq. cm. per mg. (indicated purity, 87 per cent). A considerably greater amount of starting material (600 mg.) than in the previous experiment (Fig. 1) was used in order to test the feasibility of a larger scale procedure for the isolation of DPN. The experiment was carried out at 6°.

After twenty-four transfers, the contents of each tube were withdrawn from the machine, the phenol and chloroform removed by three extractions with 15 ml. of ether, and the resulting twenty-five aqueous solutions analyzed by a determination of optical density at 260 $m\mu$. The results of this analysis are given in Fig. 2. The main band (Tubes 7 to 20), which represented DPN, was not the symmetrical type usually obtained in the counter-current distribution of a single substance but showed a precipitous rise from Tubes 7 to 10 and a more gradual decline from Tubes 12 to 19. Previous experience has shown that this type of skewed curve does not indicate the presence of impurities but is the result of a non-linear partition isotherm; i.e., a shift in distribution coefficient with concentration. In view of the symmetry of the main band in the experiment shown in Fig. 1, when a relatively small amount of starting material was used (202 mg.), it was likely that the asymmetry of the band in Fig. 2 resulted from the high initial DPN concentration. A method has not as yet been devised for the calculation of theoretical curves for substances with non-linear partition isotherms. On the other hand, it has been found that the total width of the base of a skewed curve obtained with a pure substance is very nearly the same as that of the symmetrical theoretical curve. On inspection of the two curves in Fig. 2, the only detectable impurity in the main band on the basis of absorption measurements at 260 $m\mu$ occurred in Tubes 16 to 20. This impurity corresponded to that in Tubes 13 to 17 of Fig. 1. A small amount of impurity absorbing at 260 $m\mu$ was present in Tubes 21 to 24 (Fig. 2).

In order to compare the purity of the DPN on both sides of the band shown in Fig. 2, the material in Tubes 8 to 12 and in Tubes 13 to 18 was pooled separately, frozen, lyophilized, redissolved in 5 ml. of water, and precipitated by the addition of 10 volumes of absolute alcohol. Each precipitate was washed with ether and dried in a vacuum desiccator over P_2O_5 . 220 mg. of DPN were isolated from Tubes 8 to 12, the extinction

coefficient at $340\text{ m}\mu$ after reduction with hydrosulfite being $8.36\text{ sq. cm. per mg.}$ (indicated purity, 98 per cent). Tubes 13 to 18 yielded 209 mg. of DPN, of which the extinction coefficient at $340\text{ m}\mu$ after reduction was $8.26\text{ sq. cm. per mg.}$ (indicated purity, 97 per cent). Both preparations were amorphous but perfectly white in color. The slightly lower extinction coefficient of the DPN from Tubes 13 to 18 was probably due to

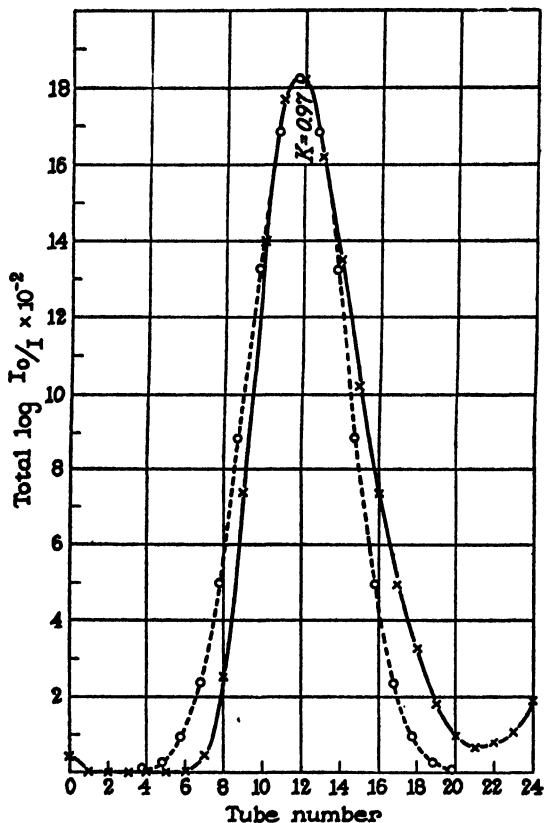


FIG. 2. Counter-current distribution of 600 mg. of partially purified DPN in the system water-phenol-chloroform. \times , total optical density at $260\text{ m}\mu$; \circ , theoretical distribution of a single substance possessing a distribution coefficient of 0.97.

small amount of impurity in Tubes 16 to 18. Reprecipitation of the DPN from Tubes 13 to 18 did not produce any change in its extinction coefficient. On the basis of extinction coefficients at $340\text{ m}\mu$, a total of 80 per cent of the DPN in the starting material was recovered.

Procedure for Isolation of DPN on Large Scale—It was apparent from the data presented above that the use of an initial DPN concentration of much

more than 3 per cent in the water-phenol-chloroform system would result in an excessive shift in the distribution coefficient. In larger scale procedures, it would therefore be necessary to increase the volume of the solvents above the capacity of the present counter-current distribution machine. The data also indicated, however, that the number of transfers required to effect satisfactory purification was sufficiently small to render the fractionation method feasible without resorting to the machine. Accordingly, the following experiment was carried out in order to test the practicability of a larger scale procedure for the isolation of DPN. Although only 1 gm. of cozymase was available for fractionation, it was evident that larger amounts could have been used if appropriate increases had been made in the volume of the solvents.

The first step in the purification was a four transfer distribution at 6° of 995 mg. of cozymase in the system, 12 ml. of water-12 ml. of phenol. The water phase contained 0.10 mg. of KCl per ml. Heavy glass-stoppered test-tubes were used for the distribution. After each equilibration, the tubes were centrifuged because of the tendency for the impurities possessing a high distribution coefficient to cause emulsification. The upper layers were transferred from one tube to the next by a vacuum-operated siphon. Tubes 0, 1, and 2 contained practically all the DPN because of its low distribution coefficient ($K = 0.12$) in the water-phenol system. Tubes 3 and 4 contained the bulk of the impurities of high distribution coefficient, corresponding to the material in Tubes 18 to 24 of the distribution shown in Fig. 1. The contents of each tube were then extracted three times with 15 ml. of ether. The resulting aqueous solutions obtained from Tubes 0, 1, and 2 were combined, frozen, and lyophilized. The residue was dissolved in 10 ml. of cold water, precipitated by the addition of 100 ml. of cold absolute alcohol, recovered by centrifugation, washed with ether, and dried *in vacuo* over P_2O_5 . This preparation of partially purified DPN consisted of yellow resinous material and weighed 684 mg. After reduction with hydrosulfite its extinction coefficient at 340 $m\mu$ was 7.14 sq. cm. per mg. Since the extinction coefficient of the original cozymase was 5.25 sq. cm. per mg., the yield in terms of DPN recovered was 92 per cent. The purity of the DPN had been increased from 62 to 84 per cent.

The aqueous solutions from Tubes 3 and 4 were evaporated to dryness at 100°. Tube 3 contained 76 mg. and Tube 4, 144 mg. of impurity.

The second step in the isolation procedure consisted in distributing in the water-phenol-chloroform system the partially purified DPN obtained from Tubes 0, 1, and 2. This distribution was carried out under conditions similar to those of the experiment shown in Fig. 2. The number of transfers, however, was reduced to sixteen. 453 mg. of DPN were collected from Tubes 4 to 11, the extinction coefficient at 340 $m\mu$ being 8.25 sq. cm. per mg. after reduction with hydrosulfite. The indicated purity of the

compound was therefore 97 per cent. The over-all yield of DPN obtained by the two distributions of 995 mg. of cozymase was 72 per cent.

Chemical Analysis of Purified DPN—Table I shows the results of elementary analysis of samples of DPN isolated from the distributions shown in Figs. 1 and 2. Both samples had been stored in a desiccator over P_2O_5 before analysis. Residual water was determined by heating the samples to constant weight at 100° *in vacuo*. In order to estimate the extent of contamination by KCl, chlorine analyses were carried out in addition to analyses for C, H, N, and P. It can be seen from the data in Table I that both samples gave low C, N, and P and high H values. Sample 2, which was purer than Sample 1 on the basis of extinction coefficients at $340 m\mu$ ($E = 8.36$ versus 8.13 sq. cm. per mg.), gave C:N and N:P ratios in exact agreement with the theory, whereas the C:N ratio for Sample 1 was

TABLE I
*Analysis (Per Cent) of Samples of Purified DPN**

Sample	Residual water	C	H	N	P	Cl	C:N	N:P
Calculated for $C_{21}H_{27}N_7P_2O_{14}$ (mol. wt., 663.5)		38.01	4.10	14.78	9.35		2.572	1.58
Found, Sample 1†	5.46	37.75	4.44	14.38	9.08	Trace	2.62	1.58
“ “ 2‡	5.30	37.33	4.40	14.52	9.18	0.10	2.571	1.58

* Analyses were performed by Mr. D. Rigakos and Miss Theta Spoor of The Rockefeller Institute for Medical Research and by Dr. A. Elek, 4763 West Adams Boulevard, Los Angeles 16, California.

† DPN isolated from Tubes 6 to 12 of the distribution shown in Fig. 1.

‡ DPN isolated from Tubes 8 to 12 of the distribution shown in Fig. 2.

somewhat high. In general, the analytical values indicated that an appreciable amount of residual water was present in the samples even after they were dried at 100° *in vacuo*. Attempts to attain a completely anhydrous state were unsuccessful, however, because DPN decomposed when heated at temperatures higher than 100° .

Activity of Purified DPN As Coenzyme—As a final check on the extent of purification of DPN, it was desirable to compare the activity of the purified DPN as a coenzyme with that of a crude cozymase preparation. The lactic acid dehydrogenase system of rat liver was found to offer a convenient method for estimating DPN by enzyme assay. This determination was based on the finding that the DPN-cytochrome *c* reductase activity of rat liver homogenates was much higher than the lactic acid dehydrogenase activity.⁴ When sufficient cyanide was added to inhibit cytochrome

⁴ Hogeboom, G. H., unpublished work.

oxidase, a determination of lactic acid dehydrogenase activity could be made by following spectrophotometrically at $550\text{ m}\mu$ the rate of reduction of cytochrome *c*. Under the conditions noted below the reaction proceeded linearly with time for approximately 10 minutes, and the rate was proportional to the DPN concentration until relatively large amounts of DPN had been added.

Fig. 3 shows a comparison of the coenzyme activity in the lactic acid dehydrogenase system of a cozymase preparation ($E_{40} = 5.3$ sq. cm. per mg., indicated purity 62 per cent) with that of purified DPN ($E = 8.36$

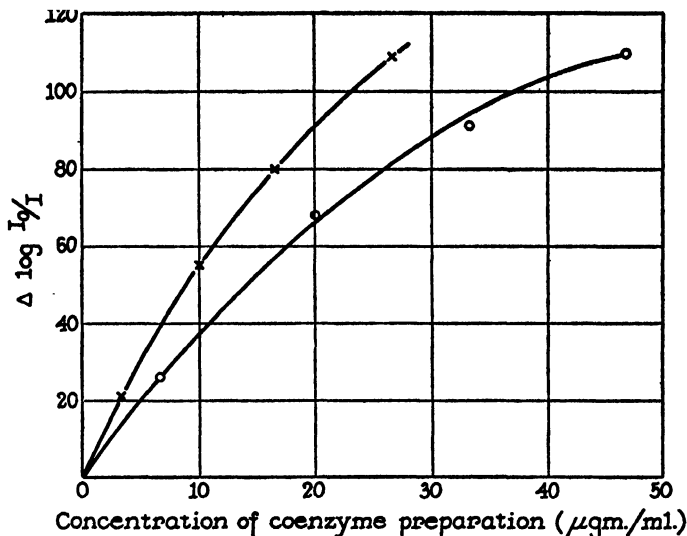


FIG. 3. Comparative activity of crude cozymase and purified DPN as hydrogen carriers in the lactic acid dehydrogenase system of rat liver. X, increase in optical density at $550\text{ m}\mu$ over a 5 minute period on the addition of increasing amounts of purified DPN; O, increase in optical density at $550\text{ m}\mu$ over a 5 minute period on the addition of increasing amounts of crude cozymase.

sq. cm. per mg., indicated purity 98 per cent). The reaction mixtures were made up by adding the following components in order: 0.20 ml. of 0.50 M $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, pH 7.4; 0.20 ml. of 0.82 M nicotinamide; water to give a final volume of 3.00 ml.; 0.30 ml. of liver extract, diluted 1:50 with 0.88 M sucrose; 0.30 ml. of 10^{-3} M NaCN; 0.40 ml. of 2.2×10^{-4} M oxidized cytochrome *c*; varying amounts of DPN or cozymase dissolved in 0.05 M potassium phosphate buffer, pH 7.4; and 0.20 ml. of 0.11 M sodium lactate. The control reaction mixture contained all components except DPN.

The liver extract was prepared as described previously (14) by homogenizing 1 gm. of rat liver in 9 ml. of 0.88 M sucrose and centrifuging the

homogenate for 20 minutes at $600 \times g$ in order to remove residual intact cells and free nuclei. The cell-free supernatant was used as a source of lactic acid dehydrogenase and DPN-cytochrome *c* reductase. Cytochrome *c* was maintained in the oxidized form by the addition of HCl to a final concentration of 0.01 M. The experiment was carried out at 22°.

After the addition of sodium lactate, the reaction mixtures were quickly transferred to cuvettes and the increase in the optical density at 550 $m\mu$ followed at intervals of a minute in the Beckman spectrophotometer. In Fig. 3 the increase in absorption over a 5 minute interval is plotted against the concentration of DPN or cozymase. It can be seen that at each level of enzyme activity the approximate ratio of crude cozymase to purified DPN yielding an equivalent reaction rate was 1:0.63. The results of this experiment are in agreement with the DPN content of the two preparations, as calculated from the extinction coefficients at 340 $m\mu$ after reduction with hydrosulfite. The experiment also demonstrated that the DPN isolated by the counter-current distribution method is active as a coenzyme.

DISCUSSION

Purity of DPN Isolated by Counter-Current Distribution—On the basis of several criteria of homogeneity, it is probable that the DPN obtained by the counter-current distribution of crude preparations of cozymase contained, aside from residual moisture, no more than a few per cent of impurities. Although not an entirely reliable measure of purity, the extinction coefficient of the DPN at 340 $m\mu$ after reduction with hydrosulfite closely approached maximum values expected for pure DPN reduced under similar conditions. In this respect, Drabkin (15) has shown that the occurrence of either transformation or reoxidation during the reduction of DPN in solution results in significantly lower extinction coefficients than corresponding values obtained for reduced diphosphopyridine nucleotide ($DPNH_2$) isolated by Ohlmeyer's procedure (16). The extinction coefficient at 340 $m\mu$ for pure anhydrous $DPNH_2$ is 9.43 sq. cm. per mg. (16). On the basis of Drabkin's data, the value for pure anhydrous DPN, reduced in solution with hydrosulfite, was estimated to be approximately 8.8 sq. cm. per mg. The extinction coefficients of the preparations of DPN isolated by counter-current distribution, after correction for residual water content, were 8.63 to 8.83 sq. cm. per mg.

Elementary analysis of the purified DPN was rendered somewhat difficult by the fact that the compound was hygroscopic and contained residual moisture after having been stored over P_2O_5 for prolonged periods in a vacuum desiccator. A completely anhydrous state was apparently not attained even after heating the compound *in vacuo* at 100°. In general, it

may be stated, however, that the analytical figures compared favorably with similar values reported in the literature for DPN considered to be pure (6, 7, 13, 17) and indicated that preparations containing at least 96 to 98 per cent DPN can be readily isolated by means of counter-current distribution.

Finally, it should be pointed out that the curves representing DPN (Figs. 1 and 2) obtained from the counter-current distribution of cozymase were in close agreement with the theoretical distribution of a single substance. It has been shown that agreement between experimental and calculated curves is an important criterion of homogeneity (9, 10, 12).

Remarks on Fractionation and Isolation Procedure—Counter-current distribution often, as in the case of the penicillins (9, 10), makes possible the separation and isolation of unstable compounds with a minimum of decomposition. In the present experiments with DPN, a substance that is easily hydrolyzed at both acid and alkaline pH, the temperature was maintained at 6° or below, and the pH of the aqueous phase of the system was approximately 5. In spite of the mildness of these conditions, however, a consideration of the curves shown in Figs. 1 and 2 brought up the possibility that a slight amount of transformation of DPN may actually have occurred. Thus the results of the distribution shown in Fig. 1 indicated that fragments of the DPN molecule containing adenine possessed a very high distribution coefficient and should have appeared almost exclusively in Tubes 23 and 24 of the experiment shown in Fig. 2. If a continuous, slow hydrolysis occurred during the distribution, however, these fragments would not be sharply localized in Tubes 23 and 24 but would extend from the right side of the DPN band to Tube 24. The fact that the total optical density of the material in Tubes 20 to 23 of Fig. 2 did not approach zero but remained almost constant could be accounted for on this basis.

Previous investigations (5) have shown that the weakest point of the DPN molecule occurs at the linkage between ribose and pyridine nitrogen and that the initial step in the hydrolysis of DPN yields free nicotinamide. In the method of isolation of DPN used in the present experiments, involving precipitation from aqueous solution with ethanol, free nicotinamide would be eliminated from the final preparation because of its solubility in ethanol. Hydrolytic products from the remainder of the molecule might be precipitated by ethanol and thus could occur as possible contaminants.

It should also be pointed out that heavy metals, which might possibly be present in small amounts in crude DPN, would remain in the aqueous phase of the water-phenol system and therefore would not contaminate DPN purified by counter-current distribution.

A comparison of the distribution coefficient of DPN and triphospho-

pyridine nucleotide (TPN) and an investigation of the behavior of the latter substance on counter-current distribution would be of considerable interest. TPN was not available in sufficient amounts, however, to permit such a study.

The authors are indebted to Dr. Lyman C. Craig for his interest and helpful suggestions.

SUMMARY

A method, based on counter-current distribution, is described for the fractionation of crude preparations of diphosphopyridine nucleotide (DPN). DPN of high purity (at least 96 to 98 per cent pure) was readily isolated in yields of 70 to 80 per cent by distribution in a two-phase system consisting principally of phenol and water. The procedure was demonstrated to be suitable for the isolation of pure DPN on a large scale.

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SERUM TREATMENT OF WESTERN EQUINE ENCEPHALITIS IN MICE DETERMINED BY THE COURSE OF VIRAL INFECTION

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Certain features of serotherapy of experimental infections with neurotropic viruses are still under deliberation. A group of investigators maintain that animals prostrated by the infection can still be completely revived and restored to a normal-seeming state while other workers hold that once the virus multiplies actively or the host exhibits a definite syndrome reflecting the involvement of the central nervous system, *i.e.*, a destruction of tissue or neurons, then serotherapy is often ineffective. There is general agreement, however, on the successful prevention or treatment of the experimental disease if potent antiserum in sufficient amount is given early enough in its course, or, generally, before definite physical signs of infection of the central nervous system are manifest.

The problem was to determine at what stage during the course of an experimental infection antiserum was or was not effective, especially in relation to the a) time after exposure to the virus, b) first appearance of physical signs and c) degree of multiplication of the virus in the brain. By means of the intracerebral route, 100% rate of lethal infection could be obtained with a sufficient dose of virus; and by the use of the Kelser strain of Western equine encephalitis virus which is only moderately active or invasive, the incubation period could be conveniently prolonged for serum treatment at different intervals after exposure to virus. Mice were the animals of choice since large numbers could be studied thus avoiding statistical pitfalls and yet permitting the conservation of a supply of the rabbit hyperimmune serum used in the present investigation. The blood volume of a mouse of about 17 g weight—the average weight of the test mice—is 1 cc; since 2 cc of the serum was given each animal, it was therefore the equivalent of twice the animal's blood volume.‡ More than 2 cc of this type of serum was toxic by itself, reaching a rate of 100% of deaths within a few hours after 5 cc were given.

The rate of multiplication of the Kelser strain had been studied¹ by sacrificing

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† We are grateful for the technical assistance of Edna Blinder and Joan B. Fitzgerald.

‡ The blood volume was calculated from the formula of Dreyer, G., and Ray, W., (*Philos. Trans. Roy. Soc., London, 1911, Series B, 201, 133*) *vis.*, $B.V. = \frac{\text{Weight} \frac{2}{3}}{6.70}$.

¹ Schlesinger, R. W., unpublished observations made in this laboratory.

mice at 4-hour intervals from 2 to 94 hours after intracerebral inoculation of 2700 LD₅₀ of the virus. As seen in Table I, the multiplication of the virus proceeded at a fairly regular rate between the 10th and 54th hour after inoculation. After about 57 hours, the virus reached its maximum titer which it maintained until the time of death. On the other hand, visible signs of illness were first noted 74 hours after inoculation and deaths occurred at the 86th to 96th hour, or later.

TABLE I

Multiplication of Western Equine Virus (Kelser Strain) in Mouse Brain after Intracerebral Inoculation of about 2700 LD₅₀

Hr after inoculation	Virus titer of brain tissue removed from inoculated mice*	Hr after inoculation	Virus titer of brain tissue removed from inoculated mice*
2	$<2 \times 10^{-1.0}$	50	$2 \times 10^{-8.3}$
6	$2 \times 10^{-1.5}$	54	$2 \times 10^{-7.0}$
10	$2 \times 10^{-2.0}$	57	$2 \times 10^{-7.4}$
14	$2 \times 10^{-2.5}$	62	$2 \times 10^{-7.5}$
18	$2 \times 10^{-4.0}$	66	$2 \times 10^{-7.3}$
22	$2 \times 10^{-4.5}$	70	$2 \times 10^{-7.5}$
26	$2 \times 10^{-4.5}$	74	$2 \times 10^{-8.3}$
30	$2 \times 10^{-5.4}$	76	$2 \times 10^{-7.5}$
33	$2 \times 10^{-6.3}$	80	$2 \times 10^{-7.0}$ †
38	$2 \times 10^{-6.5}$	86	$2 \times 10^{-7.3}$ ‡
42	$2 \times 10^{-6.5}$	90	$2 \times 10^{-7.0}$ ‡
46	$2 \times 10^{-6.4}$	94	$2 \times 10^{-7.0}$ ‡

* Brain removed aseptically; diluted with 10% normal rabbit serum in saline solution and injected intracerebrally in test mice. The LD₅₀ is given in the table.

† Signs of central nervous system involvement in this period.

‡ Death due to virus in this period.

Thus a base line was constructed of a) the rate of multiplication of the virus in the brain of inoculated mice, b) the time of recognizable physical signs of infection and c) the time of death of the animal. The plan was to treat infected mice with antiserum at different periods of infection, for example, when no signs were visible and no multiplication of the virus occurred; when no signs were noted but multiplication was active, and when signs of illness were plainly seen. In this way a correlation of the effectiveness of the antiserum with the degree of multiplication of virus and of recognizable signs of infection could perhaps be obtained.

Preparation of Antiserum. At first rabbits were immunized by intraperitoneal injection of mouse-brain virus in amounts representing at least 10^{7.5} LD₅₀. For example, after 6 such injections a rabbit antiserum when diluted 1:100 exhibited a neutralizing index of 500, as shown by the intracerebral

mouse-neutralization test. After an added course of 3 inoculations of mouse-brain virus, 1:1000 dilution of the rabbit antiserum completely neutralized 65 LD₅₀ of virus, and 1:5000 and 1:10,000 dilution of serum still showed neutralizing effect, although not completely (2 of 5 mice died) against the same amount of virus. Beyond this point, further treatment failed to raise the level of neutralizing antibody. This is in good agreement with a prior conclusion that immunization prolonged over a period of months does not necessarily lead to antibody levels higher than those obtained by properly administered shorter courses.² However this may be, rabbit antiserum prepared by means of mouse-brain virus could not be used owing to the toxicity of such prodigious amounts as were given to test animals; *viz.*, twice the blood volume. Nontoxic rabbit serum was then prepared by employing as immunizing antigen embryo-nated hens' eggs infected via the yolk sac with the Kelser strain of Western equine encephalitis virus. || After 9 intraperitoneal injections of "egg" virus having a titer of 10^{-7.5} or higher, given over a period of a month, the pooled serum from 4-6 rabbits exhibited about the same degree of viral neutralization as antiserum prepared against mouse-brain virus just described. In a test, however, serum was used which in a dilution of 1:500 or higher could prevent infection of 5-25 lethal doses of virus injected intracerebrally into mice. This preliminary titration was made by inoculation of 1 cc of graded dilutions, up to 1:1000, of antiserum subcutaneously and followed 16 hours later by injection of a constant amount, 10^{-5.8}-10^{-6.0}, of virus. A control titration of the virus by itself was included so that an exact computation of the number of doses used in the test could be obtained.

Tests. Table II serves to demonstrate examples of the tests in which mice were injected with varying small multiples of lethal doses of the virus of Western equine encephalitis and given antiserum either before, along with, or after inoculation of the virus. Each mouse received .03 cc of the virus suspension intracerebrally and 2 cc of antiserum were administered intraperitoneally. A series of mice were also treated with similar injections of normal rabbit serum as controls but as the table shows, no influence of the normal serum was exerted on the course of the experimental infection. It will be noted that the number of infective units of virus used in the test was less than that employed for determining the rate of multiplication of the virus in the brain of virus-inoculated mice and the time of development of physical signs and death in them, the data of which are shown in Table I. This was purposely planned so that the

² Olitsky, P. K., Schlesinger, R. W., and Morgan, I. M., *J. Exp. Med.*, 1943, 77, 359.

|| In preparing the immunizing antigen in large amounts, contamination could be reduced or eliminated by placing harvested embryos in a saline solution containing 1000 units penicillin per cc. The embryos were kept in the solution until the required number was collected and were then transferred to a Waring Blendor.

reactions to the virus by the serum-treated animals would not be greater than those in these control mice, and furthermore, the smaller doses of virus used in the test animals would weigh in favor of any preventive or curative action of the antiserum. For it will be seen (Table II) that the antiserum even in the large quantities given became less pronounced in effect when the virus was increased from 5 to 25 LD₅₀, an outcome that was not unexpected. The

TABLE II

Treatment with Hyperimmune and Normal Rabbit Serum of Mice Infected by Intracerebral Route with Varying Amounts of Virus

Hyperimmune rabbit serum					Normal rabbit serum†
Virus titer	10 ^{-6.7}	10 ^{-7.3}	10 ^{-7.7}	10 ^{-7.8}	
Dilution to infect . . .	10 ^{-6.0}	10 ^{-6.8}	10 ^{-6.0}	10 ^{-6.8}	
LD ₅₀ used	5*	25	50	63	
Time when antiserum was given in relation to virus	Result Dead/No. used				Result Dead/No. used
2 hr before	—	0/6	—	—	5/5
1 " "	1/7	—	—	—	—
With virus	2/6	0/6	—	1/5	9/9
4 hr after	0/7	1/6	0/5	—	4/4
8 " "	—	2/6	—	—	5/5
16 " "	0/6	2/6	2/5	—	9/9
24 " "	3/7	5/6	—	4/6	9/9
30 " "	2/7	6/6	4/6	3/5	9/9
48 " "	4/6	6/6	3/6	6/6	9/9
72 " "	5/7	6/6	6/6	—	5/5

* Antilog of difference between lines 1 and 2 to nearest whole number.

† 2 cc of normal rabbit serum was given intraperitoneally to each of a series of animals receiving intracerebrally 25 and 63 LD₅₀ of virus and the results of both tests are combined here.

increase of inoculated virus from 25 to 63 doses, however, exerted little if any influence on the action of the antiserum.

There was, in addition, variation in the effect of antiserum within the same series. This variation is found now and again in immunity tests of this sort and is perhaps an indicator of the varying but unpredictable individual factors of resistance that may be brought out by the action of hyperimmune serum, such as the rate of aging, physiological or constitutional elements, or other still unknown influences which would help certain animals.

It will be observed that the antiserum became less effective as the time after exposure to the virus lengthened. Thus, up to 16 hours after the virus was

given, encephalitis could generally be prevented in treated mice. From 24 to 48 hours after the inoculation of virus, some but not all the treated animals remained apparently unaffected. At 72 hours, at a time when the mice were still free from signs of illness, only an occasional test animal receiving the minimal amount of virus survived. When the same amount of antiserum was given to animals when definite physical signs of illness occurred, at a time corresponding to 73 or more hours after exposure to minimal amounts, *i.e.*, about 5 or 6 LD₅₀ virus, the treatment proved valueless since all treated mice died (Table III).

TABLE III
*Serum Treatment of Mice at the Time of First Definite Signs of Illness**

Mouse No.	LD ₅₀ of virus injected	First sign shown after virus inoculation	Signs shown after virus inoculated in hours
1	ca. 6	Leg weakness	73
2	"	Tail spastic	73
3	"	Ruffled fur; slow	90
4	"	" " tremors	90
5	"	" " slow	90
6	"	Circling	93
7	"	Biting (itch)	93
8	"	Ruffled fur; slow	93
9	"	" " "	93
10	"	" " "	96
11	5	" " "	90
12	5	" " "	93

* All animals died after administration of 2 cc of antiserum given *iper.* at the time of the appearance of physical signs.

Discussion. There is no expediency for the acceptance of the pattern laid here for serum treatment of mice as a model for that of human beings. The infection was induced in mice by intracerebral inoculation of the virus of Western equine encephalitis which is not the way of infection in nature. The effect of serotherapy in the human disease is at present not definitely known since no controlled series of observations are as yet available; Hammon³ states that convalescent serum therapy is probably ineffective.

In the present study mice and the intracerebral route of infection have been employed for the purpose of providing sufficiently large numbers of conveniently handled animals for several tests, and secondly, to have a more solid foundation on which to discern real and not apparent results since the intracerebral route could be counted upon to induce lethal encephalitis in every instance among controls. It therefore follows that what was concluded from the experiments

³ Hammon, W. M., *Clinics*, 1945, 4, 485.

with mice and the intracerebral route of inoculation might not correspond with the results of tests on larger laboratory animals, such as guinea pigs and monkeys, and on the use of peripheral (nonneural) route of inoculation of virus. Then, of necessity, smaller numbers of animals have to be used and the peripheral route of viral exposure is not always disease-producing in the control animals.

What is significant is that in the present investigation a certain dosage of antiserum which could prevent infection at one particular stage during the course of infection, when the virus was even actively multiplying, was ineffective in another, when virus was increasing, or remained stationary at a higher level. Either the antiserum was incapable of neutralizing the virus at its place of multiplication⁴ or an amount of serum might have been needed that in terms of blood volume of the host was impractical to use, or changes in the tissues progressed to a degree which could no longer be influenced by serotherapy. It should be stressed, however, that the stage during which antiserum was ineffective was one in which the treated animals could still appear to be normal to the observer.

Summary. If 2 cc of rabbit hyperimmune antiserum of high titer were given intraperitoneally to mice of about 17 g weight—a prodigious amount since it represents twice the total blood volume of the host—encephalitis was prevented from developing in animals receiving intracerebrally ordinarily lethal doses of the virus of Western equine encephalitis. The preventive effect was noted when the virus was multiplying but had not as yet reached its maximal titer. Thus, if animals were treated with antiserum 2 hours before, at the same time, or from 4–16 hours after the virus was inoculated, it was effective in preventing an attack of encephalitis. Even if serum was administered 24, 36 or 48 hours after the virus certain but not all mice survived without recognizable signs of the disease. At 72 hours, however, only an exceptional mouse survived after serum treatment. Finally, at 73 or more hours after virus inoculation, when definite manifestations of experimental Western equine encephalitis could be observed, injection of antiserum was found to have no influence on the course of the lethal infection.

⁴ Rivers, T. M., *J. Am. Med. Assn.*, 1948, 136, 291.

THE EFFECT OF SPHINGOMYELIN ON THE GROWTH OF TUBERCLE BACILLI

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The enhancing effect of the synthetic wetting agents Tween 40, Tween 60, and Tween 80 on the growth of tubercle bacilli is due in part to the fact that these water-dispersible esters constitute for the bacteria a non-toxic source of palmitic, stearic, or oleic acid. Indeed, enhancement of growth can also be observed with the sodium soaps of these same fatty acids, as well as of linoleic, linolenic, and arachidonic acids, provided that the bacilli are protected against the toxic action of these soaps, for example by the addition of serum albumin to the medium (6).

In addition to long chain fatty acids, there exist in tissues other lipids which enhance the growth of tubercle bacilli (1, 2, 5, 10). Further study of this problem has led to the recognition that, in this respect, sphingomyelin is more active than any of the other naturally occurring phospholipids and cerebrosides tested in our laboratory. It will be shown in the present paper that the addition to simple culture media of sphingomyelin prepared from various tissues markedly increases the density of growth within a short period of incubation and also facilitates initiation of growth from small inocula, even in the absence of serum albumin or other protein.

EXPERIMENTAL

All experiments described in the present report were carried out with a virulent culture of the strain of human tubercle bacillus H37Rv recently recovered from infected mouse lung and maintained in the Tween-albumin medium described elsewhere (8). The 10 day old culture used for inoculation consisted of finely dispersed bacilli and contained approximately 0.35 mg. bacillary cells (dry weight) per cc. of medium.

The basal medium used in the experiments had the following composition:

KH ₂ PO ₄	1.0	gm.	} heat in 100 cc. distilled water to dissolve
Na ₂ HPO ₄ ·12 H ₂ O.....	6.3	"	
Asparagine.....	1.0	"	
Add:			
Distilled water.....	850 cc.		
Enzymatic digest of casein....	0.5	gm.	(10 cc. of a 5 per cent solution in distilled water)
ZnSO ₄	0.001	"	(1 cc. of a 0.01 per cent solution in distilled water)
Ferric ammonium citrate.....	0.005	"	
MgSO ₄ ·7 H ₂ O.....	0.01	"	(1 cc. of a 1 per cent solution in distilled water)

CaCl ₂	0.0005 gm.	(1 cc. of a 0.05 per cent solution in distilled water)
CuSO ₄	0.0001 "	1 cc. of a 0.01 per cent solution in distilled water)

Adjust pH to 6.5

It was distributed in 3 cc. amounts in pyrex glass tubes 25 mm. in diameter. For reasons given in an earlier publication, aluminum caps were used instead of cotton plugs (7). The other ingredients were added, as indicated in the text, either before autoclaving or aseptically as sterile solutions.

Serum albumin was the fraction V of bovine plasma. It was dissolved in a concentration of 5 per cent distilled water. The solution was sterilized by filtration through a porcelain candle.¹

The non-ionic wetting agent Triton A20² was added to the medium in a number of tests. This wetting agent is a heat-stable arylalkyl polyether of phenol which disperses the cultures of tubercle bacilli without increasing the yield of growth. Its properties will be further discussed in a subsequent publication.

Most of the preparations of sphingomyelin, cerebroside, lignoceric amide, lignoceric acid and sphingosine used in the present study were obtained from the chemical collection of the late Dr. P. A. Levene maintained at the Rockefeller Institute. The chemical structure and properties of these substances as well as the methods involved in their preparation have been described in references 9 and 11. A sample of pure lung sphingomyelin was generously supplied by Dr. S. J. Thannhauser of Joseph H. Pratt Diagnostic Hospital, Boston; this material had been freed of contaminating dipalmityl glyceride by the technique described in reference 12.

All preparations of lipids were dispersed in distilled water starting from chloroform solutions whenever necessary. Sphingomyelins and cerebroside were sterilized by three consecutive heatings at 80°C., although more recent tests indicate that their effect on the growth of tubercle bacilli is not appreciably modified by autoclaving in the basal medium.

All lipids imparted a certain degree of opalescence to the medium. It was observed that complete dispersion of sphingomyelin, resulting in a clear medium, could be obtained by adding to the medium the wetting agent Triton A20 (see above) in a final concentration of 0.01 to 0.02 per cent.

Effect of Sphingomyelin and Cerebroside Preparations on the Growth of Tubercle Bacilli.—The yield of bacilli within a given period of incubation and the ability of the medium to allow proliferation of very small inocula have been used as criteria in the following experiment to compare the growth-promoting properties of sphingomyelins and cerebroside and of serum albumin.

The preparations of sphingomyelin and cerebroside to be tested were added in 0.3 cc. amounts of emulsions of various concentrations to 3 cc. of the basal medium. Albumin (0.3 cc. of 5 per cent solution), or water, was used for the control media. Four sets of each medium were prepared; to two had been added 0.02 per cent Triton A20 prior to autoclaving. The tubes were inoculated with 0.003 or 0.000003 cc. of a 10 day old culture of H37Rv in Tween albumin medium, diluted in 0.3 cc. distilled water. These inocula corresponded approximately to 3×10^{-4} and 3×10^{-7} mg. of dry bacilli per cc. of medium. Final readings of

¹ Bovine albumin (serum fraction V) was obtained in a desiccated form from Armour Laboratories, Chicago.

² Triton A20 was generously supplied by Rohm and Haas Company, Philadelphia.

macroscopic evidence of growth, confirmed in some cases by microscopic examination, were made after 10 days' incubation at 37°C.

TABLE I

Comparative Effects of Sphingomyelins, Cerebrosides, and Serum Albumin on the Growth of Tubercle Bacilli

Test substances added to the medium			Inoculum H37Rv (mg. dry bacilli per cc. medium)			
			3×10^{-4}		3×10^{-7}	
			Basal medium	Basal medium + 0.02 per cent Triton A20	Basal medium	Basal medium + 0.02 per cent Triton A20
	source*	Final concentration <i>per cent</i>				
Kidney sphingomyelin	P.A.L. (1126)	0.05	5†	7†	2†	3†
		0.01	3	5	1	2
Liver	" (1127)	0.05	6	8	2	2
		0.01	4	6	2	3
Brain	" (1131)	0.05	5	7	2	3
		0.01	3	5	1	3
Lung	Thannhauser	0.05	5	7	2	3
		0.01	3	5	2	3
Cerebron	P.A.L. (928)	0.05	2	2	0	0
		0.01	3	5	2	3
Cerebron	" (932)	0.05	1	0	0	0
		0.01	2	2	0	0
Phrenosin	" (1115)	0.05	2	1	0	0
		0.01	2	1	0	0
Serum albumin		0.5	7	8	3	4
H ₂ O			2	2	0	0

* The initials P.A.L. indicate that the material was obtained from the chemical collection of the late Dr. P. A. Levene maintained at the Rockefeller Institute. The number in parentheses refers to the classification number in this collection. The sample of lung sphingomyelin was received from Dr. S. J. Thannhauser of Boston.

† The amount of growth is indicated in terms of an arbitrary ascending scale (from 0 to 8) based on gross macroscopic examination. The figure 8 corresponds to approximately 0.4 mg. dry bacilli per cc. of medium.

The results presented in Table I show that, after 10 days' incubation, growth in the absence of either serum or lipid could be detected only in the tubes having received the larger inoculum. At that time, growth was abundant in all the

albumin media irrespective of the size of the inoculum. The cerebroside preparations did not improve, and in fact decreased, the ability of the medium to support the growth of the small inocula. All preparations of sphingomyelin, on the contrary, were almost as effective as serum albumin in enhancing the yield of bacilli and in allowing the proliferation of the small inocula. Addition to the medium of the wetting agent Triton A20 improved the performance of sphingomyelin on several accounts: (a) it dispersed the phospholipid, thereby rendering the medium more limpid; (b) it increased the bacillary yield; (c) it

TABLE II

Comparative Protective Effects of Sphingomyelin and Albumin against the Toxicity of Fatty Acids

Final concentration in basal medium of			Growth 10 days after inoculation with 3×10^{-4} mg. H37Rv per cc. medium
Fatty acid per cent	Albumin per cent	Sphingomyelin per cent	
Oleic acid 0.03	0	0	0*
" " "	0	0.1	7
" " "	0.5	0	8
" " 0.01	0	0	0
" " "	0	0.1	7
" " "	0.5	0	7
Lauric acid 0.01	0	0	0
" " "	0	0.1	5
" " "	0.5	0	6
Capric acid 0.01	0	0	0
" " "	0	0.1	5
" " "	0.5	0	6
H ₂ O	0	0	3
"	0	0.1	5
"	0.5	0	6

* Symbols same as in Table I.

caused the growth to be fairly well dispersed (diffuse growth) instead of being granular as was the case in media containing either albumin or lipids without the wetting agent.

The Neutralizing Effect of Sphingomyelin on the Toxicity of Long Chain Fatty Acids.—As shown in earlier publications, serum albumin promotes the proliferation of small inocula of tubercle bacilli not necessarily by supplying nutritive factors, but rather by protecting the organisms against the toxic effect of various injurious agents (3, 4, 7). The following experiment reveals that sphingomyelin can exert a similar protective effect against the toxicity of long chain fatty acids.

The sodium soaps of caproic, lauric, or oleic acid were added to the basal medium in the concentrations indicated in Table II. Different samples of sphingomyelin were added in

amounts of 0.3 cc. of 1 per cent emulsion per 3 cc. of medium, in comparison with 0.3 cc. of 5 per cent albumin or 0.3 cc. of water. All tubes were inoculated with 0.003 cc. of H37Rv culture in Tween-albumin medium diluted in 0.3 cc. distilled water (corresponding approximately to a final inoculum of 3×10^{-4} mg. dry bacilli per cc. of medium). Macroscopic evidence of growth was read after 10 days' incubation at 37°C. The results are recorded in Table II (the data refer to only one of the preparations of sphingomyelin tested: P.A.L. 1127).

The results presented in Table II confirm earlier findings that the inhibitory effect of long chain fatty acids on the growth of tubercle bacilli can be neutralized by addition of serum albumin to the medium and establish that sphingomyelin exhibits a similar protective effect. It is of interest in this respect that addition of this phospholipid to emulsions of sodium soaps increases appreciably the apparent water solubility of the latter, suggesting the formation of a complex such as has been recognized to occur between fatty acids and albumin.

The Effect of Lignoceric Acid on the Growth of Tubercle Bacilli.—Sphingomyelin is a diaminophospholipid in which a C24 hydroxy acid (lignoceric acid) is combined in amide linkage with the base sphingosine, phosphocholine being esterified on the latter. We have not yet studied the effect of phosphocholine on bacterial growth. All samples of sphingosine tested so far have failed to enhance and indeed have caused a marked inhibition of growth; the nature of this inhibition will be considered in a subsequent publication. On the contrary, both lignoceric acid and its amide have been found to exert a beneficial effect on growth as shown in the following experiment.

Solutions of lignoceric acid and lignoceric amide in chloroform were added in graded amounts to the basal medium containing 0.02 per cent of Triton A20. Constant agitation while heating to eliminate the chloroform yielded a fairly stable emulsion of the lipids in the medium, despite their very low water solubility. The media were distributed in 4 cc. amounts in test tubes (25 mm. diameter) and autoclaved. Serum albumin was then added in final concentrations of 0.5 per cent to a duplicate set. The inoculum was 0.04 cc. or 0.004 cc. of a 10 day old culture of H37Rv diluted in 0.4 cc. distilled water (this corresponded approximately to inocula of 3×10^{-3} or 3×10^{-4} mg. dry bacilli per cc. of final medium). Macroscopic evidence of growth was recorded after 10 days' incubation at 37°C.

The results presented in Table III establish that, like sphingomyelin and oleic acid, lignoceric acid and its amide can enhance the growth of tubercle bacilli. The latter substances allow growth of small inocula and are therefore less toxic than oleic acid. This may be due to their very low solubility in water and, in the case of lignoceric amide, to the fact that the carboxyl group is masked.

On the basis of the information summarized in Tables II and III, it appears therefore that sphingomyelin exerts a favorable effect on growth by a dual mechanism. Like serum albumin, on the one hand, it protects the bacilli against certain toxic effects, in particular those of long chain fatty acids. On the other hand it constitutes a water-dispersible source of lignoceric acid which is available for metabolic utilization. In addition to their bearing on the

metabolism of tubercle bacilli, these facts may be of significance in the analysis of the factors which condition the proliferation of tubercle bacilli *in vivo*. Although sphingomyelin was first recognized in nervous tissue, it appears to be a constituent of many if not of all types of cells; and it is probable that its release from these cells at the site of infection and in caseous material would not be without effect on the growth of the bacilli.

TABLE III

Comparative Effects of Sphingomyelin, Lignoceric Acid and Amide, Sphingosine and Oleic Acid on the Growth of Tubercle Bacilli

Test substances added to the medium			Inoculum H37Rv (mg. dry bacilli per cc. of medium)			
			3×10^{-5}		3×10^{-6}	
			Basal medium	Basal medium + 0.5 per cent albumin	Basal medium	Basal medium + 0.5 per cent albumin
	Source*	Final concentration <i>per cent</i>				
Sphingomyelin	P.A.L. (1127)	0.02	8*	8*	3*	8*
Lignoceric acid	" (1076)	0.01	7	8	2	8
" "	" (1077)	"	7	8	2	8
" amide	" (1074)	0.012	7	8	3	8
Sphingosine	" (1140)	0.01	0	4	0	0
Oleic acid	"	"	0	8	0	6
" "		0.003	0	8	0	6
H ₂ O			4	6	0	4

* Symbols same as in Table I.

SUMMARY

All preparations of sphingomyelin tested, whatever the tissues from which they originated, were found to enhance the growth of tubercle bacilli *in vitro*. Cerebrosides were inactive in this respect.

Sphingomyelin promotes growth through two independent mechanisms:

(a) It neutralizes the toxicity of long chain fatty acids probably by forming with them inert complexes. This protective effect facilitates initiation of growth from small inocula.

(b) It supplies to the bacteria lignoceric acid (or its amide) which is utilized for growth. The base sphingosine, another component of sphingomyelin, does not favor and probably inhibits proliferation of tubercle bacilli.

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THE EFFECT OF WETTING AGENTS ON THE GROWTH OF TUBERCLE BACILLI

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It is possible to obtain finely dispersed growth of tubercle bacilli by adding to the media in which they are cultivated certain non-ionic wetting agents. The polyoxyethylene esters of oleic acid (Atlas G-2144), lauric acid (Atlas G-2124), sorbitan monooleate (Tween 80), sorbitan monostearate (Tween 60), sorbitan monopalmitate (Tween 40), and sorbitan monolaurate (Tween 20), have proven especially effective in this respect. In addition to their wetting effect on the cell, certain of the water-dispersible esters just mentioned are capable of enhancing the growth of many strains of tubercle bacilli, probably by supplying them with long chain fatty acids in a non-toxic form available for metabolic utilization (3). Unfortunately, the ester linkage in these wetting agents is susceptible to enzymatic hydrolysis by lipases, a fact which prohibits their use in media containing animal tissues or fluids rich in these enzymes. It is therefore desirable to find other types of wetting agents capable of promoting dispersed growth of tubercle bacilli and stable in the presence of animal tissues. The present report describes the properties of an arylalkyl polyether of phenol (Triton A20) which in some respects fulfills these requirements.

EXPERIMENTAL

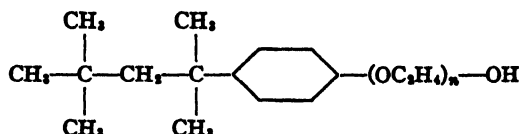
Cultures.—For the sake of brevity only experiments carried out with the human strain of tubercle bacillus H37 and with the avian strain Camden 3 will be described. Results with other strains have confirmed the findings to be presented here. Two variants of the culture H37 were used; both had been obtained originally from the Trudeau Laboratory through the generosity of Dr. W. Steenken, Jr. The virulent variant, H37Rv, was reisolated from the lung lesions of experimentally infected mice. The avirulent variant, H37Ra, was reisolated from a colony exhibiting characteristic morphology on oleic acid-albumin agar. The cultures were maintained by weekly transfers in Tween 80-albumin liquid medium and possessed the morphological characteristics of the virulent and avirulent forms, respectively (7). The avian strain Camden 3 was recently isolated in our laboratory from the liver of a tuberculous chicken and was also maintained in Tween-albumin agar. The cultures used for inoculation had been incubated for 8 to 10 days at 37°C. and contained approximately 0.35 mg. bacilli (dry weight) per cc. of medium.

Media.—The basal medium has been described in preceding reports (6); bovine serum albumin was added aseptically (in a final concentration of 0.5 per cent) after autoclaving of the medium, with or without 1.5 per cent agar. The wetting agents were added prior to autoclaving.

The sphingomyelin used was sample 1127 of the collection of the late Dr. P. A. Levene (4). It was added to the medium in the form of a 1 per cent solution in distilled water, sterilized by three consecutive heatings at 80°C.

The nature and properties of the polyethylene esters of the Tween type have been described in earlier publications (3, 5).

Triton A20 is described by the manufacturer as an arylalkyl polyether of phenol. The related compound, Triton N100, has the following general formula.



The chemical structure of Triton A20 is slightly different from that of Triton N100 and has not been published. Although both these products are related non-ionic wetting agents, Triton A20 is much less toxic than Triton N100 for tubercle bacilli and for experimental animals. For this reason, it has been selected for further study.

Triton A20 is miscible in all proportions with water and can be handled like Tween 80 in the preparation of liquid and agar media; as in the case of Tween 80, its aqueous solutions are cloudy above 80–90°C. but become limpid again at lower temperatures. Triton A20 is distributed by the manufacturer as a 20 per cent solution in water. The concentrations reported in the present publication are corrected in terms of the original material.

The media were distributed in 4 cc. amounts in Pyrex test tubes, 25 mm. in diameter. As described elsewhere, aluminum caps were used instead of cotton plugs during autoclaving and incubation (5).

Comparative Effects of Triton A20 and Tween 80 on the Growth of Tubercle Bacilli.—All cultures of tubercle bacilli so far tested are capable of giving macroscopical evidence of growth within 10 to 15 days in the basal medium to which has been added bovine serum albumin, even when the inoculum contains only a few living cells; growth in this plain albumin medium always consists of large clumps of bacilli. Tween 80 in concentration of 0.02 to 0.2 per cent increases the amount of growth and changes its character from granular to diffuse. The effect of Triton A20 was studied in the following experiment.

Tween 80 and Triton A20 were added to the basal medium in final concentrations ranging from 0.003 to 0.3 per cent as indicated in Table I. Each test medium was inoculated with 0.003 or 0.0003 cc. of a 10 day old culture in Tween albumin medium of H37Rv or H37Ra diluted in 0.3 cc. of 5 per cent bovine serum albumin. This corresponded to an inoculum of approximately 3×10^{-4} and 3×10^{-6} mg. of bacilli (dry weight) per cc. of test medium. Macroscopical evidence of growth, checked in certain cases by microscopic examination, was read after 14 days' incubation at 37°C., and is recorded in Table I.

The results presented in Table I reveal that a definite dispersing effect on the growth of virulent and avirulent variants H37Rv and H37Ra can be detected with concentrations of Tween 80 or of Triton A20 as low as 0.01 per cent. However, the dispersing effect of Tween 80 is more complete than that of the latter wetting agent. Even with the highest concentrations of Triton A20 used, the cultures of H37Rv exhibited on careful macroscopic examination a finely granular growth which was shown by microscopic study to consist of long strands of bacilli.

The two wetting agents differ also in their effect on the yield of bacilli. Addition of Tween 80 to the medium brings about a definite enhancement of growth, probably caused by the oleic acid in the Tween molecule.¹ Increase in growth does not occur with Triton A20 which apparently is not utilized by tubercle bacilli.

Finally it is obvious that whereas Tween 80 enhances to a similar degree the growth of both the H37Rv and H37Ra variants, Triton A20 behaves very differently toward the two cultures as it inhibits the growth of the latter but

TABLE I
The Effect of Tween 80 and Triton A20 on the Growth of Tubercle Bacilli

Wetting agent added to the medium		Inoculum (mg. dry bacilli per cc. of medium)			
		3×10^{-4}		3×10^{-5}	
		H37Rv	H37Ra	H37Rv	H37Ra
	per cent				
Tween 80	0.03	8*f.d.†	8 f.d.	6 f.d.	6 f.d.
“ “	0.01	8 f.d.	8 f.d.	6 f.d.	6 f.d.
“ “	0.003	6 g.	6 g.	5 g.	4 g.
Triton A20	0.3	4 d.	0	3 d.	0
“ “	0.1	5 d.	2 d	4 d.	0
“ “	0.03	5 d.	5 f.d.	4 d.	2 d
“ “	0.01	5 d.	5 f.d.	4 d.	3 d
“ “	0.003	5 g.	5 g.	3 g.	3 g.
H ₂ O		5 g.	5 g.	3 g	3 g.

* The amount of growth is recorded in terms of an arbitrary scale from 0 (no growth) to 8 (growth corresponding to approximately 0.4 mg. dry weight of bacilli per cc. of medium).

† f.d. indicates that growth was finely dispersed, exhibiting no large clumps on microscopic examination. d. indicates that the growth was dispersed but consisted of clumps readily seen with a hand lens. g. indicates granular growth consisting of large clumps or flakes.

not that of the former. It should be emphasized at this time that the toxic effect on H37Ra reported in Table I has also been observed with a number of other avirulent variants of mammalian strains of tubercle bacilli whereas none of the variants appears to be unfavorably affected by Triton A20 in concentrations of 0.05 per cent or less.

¹ It has been shown elsewhere that under certain conditions, Tween 80 can exhibit toxicity owing to its contamination with free fatty acid; the toxicity can be overcome by addition of adequate amount of serum albumin to the medium (1-3). Furthermore, we have repeatedly observed that samples of Tween which had proved entirely non-toxic when first used, develop some 2 months after the container has been opened a type of toxicity which seems to be independent of the presence of fatty acids. It is essential therefore to use only selected samples of Tween if conclusions are to be drawn concerning the effect of polyoxyethylene derivatives of sorbitan monooleate on the growth of tubercle bacilli.

The Growth-Dispersing Effect of Tween 80 and Triton A20 in Serum Media.—

It has been shown in preceding publications that incubation of Tween 80 with fraction V of plasma (serum albumin) results in the liberation of free fatty acid as a result of enzymatic hydrolysis of the ester by the lipase contaminating the albumin preparation (1, 2). In experiments previously reported, lipase activity had been minimized by heating the albumin for 30 minutes at 56°C. prior to addition to the medium. On the other hand, Tween 80 is rapidly hydrolyzed in the presence of tissues or tissue fluids and there is no convenient technique for abolishing the marked lipolytic activity of these materials. As there is no

TABLE II

The Influence of Blood Serum on the Growth-Dispersing Effect of Wetting Agents

Wetting agent added to the medium Final concentration		Serum		Growth 14 days after inoculation with H37Rv (3 × 10 ⁻⁴ mg. dry weight per cc. medium)
		Mouse	Ox	
	per cent	cc.	cc.	
Tween 80	0.05	0	0	8*f.d.*
" "	"	0.5	0	8 g.
" "	"	0	0.5	8 g.
G 2144	"	0	0	8 f.d.
" "	"	0.5	0	8 g.
" "	"	0	0.5	8 g.
Triton A20	"	0	0	5 d.
" "	"	0.5	0	8 d.
" "	"	0	0.5	8 d.
H ₂ O		0	0	5 g.
"		0.5	0	8 g.
"		0	0.5	8 g.

* Symbols as in Table I.

enzyme of animal tissues known to be capable of attacking the ether linkages of the Triton A20 molecule, it was of interest to compare Triton A20 with two water-dispersible esters of oleic acid (G 2144 and Tween 80) with respect to their ability to disperse growth of tubercle bacilli in media with or without added serum.

The wetting agents were added to the basal medium in a final concentration of 0.05 per cent. Bovine or mouse serum sterilized by filtration through porcelain candles was added in amount of 0.3 cc. per 3 cc. of medium. The tubes containing the test media were inoculated with 0.003 cc. of 10 day old culture of H37Rv diluted in 0.3 cc. of 5 per cent bovine albumin. It is known from earlier studies that this amount of albumin is sufficient to overcome the toxicity of the free fatty acid which might be released by enzymatic lipolysis of Tween 80 or G 2144. Macroscopic evidence of growth confirmed by microscopic examination was read after 14 days' incubation at 37°C. (Table II).

The results presented in Table II confirm the fact that the dispersing effect of Triton A20 on the growth of tubercle bacilli in albumin medium without serum is less complete than that of the water-dispersible esters Tween 80 or G 2144. On the other hand it is seen that in the presence of whole serum these esters completely lose their dispersing activity whereas that of Triton A20 remains unimpaired. In view of the complexity of serum and of the many unknown interactions which certainly occur between its constituents, the tubercle bacilli, and the wetting agents, it is not possible to obtain convincing evidence concerning the factors which determine the dispersed or granular

TABLE III

The Effect of Triton A20 on the Initiation and Yield of Growth of Tubercle Bacilli

Added to the basal medium (final concentration)					Growth 14 days after inoculation with (mg. cc/cc. medium)						
Tween 80	Triton A20	Oleic acid	Sphingo- myelin	Albu- min	H37Rv			H37Ra			Avian
					3×10^{-4}	3×10^{-4}	3×10^{-6}	3×10^{-4}	3×10^{-4}	3×10^{-6}	3×10^{-6}
per cent	per cent	per cent	per cent	per cent							
0	0	0	0	0	3 g.*	1 g.	0	2 g.	1 g.	0	1 f.d.
0	0	0	0	0.5	6 g.	4 g.	2 g.	7 g.	5 g.	1 g.	1 f.d.
0.05	0	0	0	0	3 f.d.	0	0	3 f.d.	0	0	5 f.d.
"	0	0	0	0.5	8 f.d.	5 d.	3 f.d.	7 f.d.	4 f.d.	1 d.	8 f.d.
0	0.05	0	0	0	3 d.	2 d.	0	1 d.	0	0	1 f.d.
0	"	0.02	0	0	0	0	0	0	0	0	0
0	"	0	0	0.5	5 d.	4 d.	3 d.	3 d.	1 d.	0	1 f.d.
0	"	0.02	0	"	8 d.	6 d.	3 d.	2 d.	0	0	6 f.d.
0	"	0	0.05	0	8 d.	5 d.	2 g.	6 d.	2 g.	1 g.	5 f.d.
0	"	0	"	0.5	8 d.	6 d.	4 d.	7 d.	5 d.	4 d.	8 f.d.
0	0	0	"	0	4 g.	3 g.	2 g.	4 g.	3 g.	2 g.	5 f.d.
0	0	0	"	0.5	7 g.	4 g.	3 g.	7 g.	4 g.	3 g.	7 f.d.

* Symbols as in Table I.

character of the bacterial growth. Nevertheless, the facts observed are consistent with the hypothesis that the water-dispersible esters Tween 80 and G 2144 are destroyed by the serum lipase and thereby lose their wetting properties, whereas Triton A20 remains unaffected under the same conditions.

The Effect of Triton A20 on the Initiation and Yield of Growth of Tubercle Bacilli.—Although Triton A20 can disperse the growth of tubercle bacilli it never increases significantly the yield of growth. However enhancement of growth in its presence can occur when long chain fatty acids, or sphingomyelin, are added to the medium.

Tween 80, Triton A20, oleic acid, sphingomyelin, and albumin were added to the basal medium as indicated in Table III. The media were inoculated with 0.003, 0.0003, or 0.000003 cc. of a 10 day old culture diluted in 0.3 cc. distilled water; these inocula corre-

sponded to approximately 3×10^{-4} , 3×10^{-4} , 3×10^{-6} mg. bacilli (dry weight) per cc. of medium. The amount and character of the bacterial growth were recorded after 14 days' incubation at 37°C.

The results presented in Table III illustrate again the striking differences between the wetting agents Tween 80 and Triton A20 with reference to their effects on the growth of tubercle bacilli. They confirm the finding of the preceding experiment that, in contrast with Tween 80, Triton A20 added to the basal medium with or without albumin does not increase significantly the amount of growth, although it has a definite dispersing effect on it.

Failure to enhance growth is particularly well demonstrated in the case of the avian strain Camden 3, a fact in agreement with earlier observations that all avian strains tested give only very limited growth in the absence of long chain fatty acids (3). However, addition of oleic acid to the albumin medium containing A20 definitely enhances growth which remains dispersed despite the presence of the fatty acid. The growth-promoting effect of sphingomyelin first demonstrated in the preceding communication (4) is here confirmed. It is shown moreover that the beneficial effect of sphingomyelin, rendered even more evident in the presence of Triton A20 which increases the solubility of this phospholipid, is expressed not only in terms of greater density of the culture at the end of the incubation period, but also by the fact that it allows the growth of minute inocula even in the absence of serum albumin (3).

Comparison of the results obtained with the virulent (H37Rv) and avirulent (H37Ra) variants illustrates again the selective inhibitory effect of Triton A20 on the growth of the latter culture. It is clear however, that the inhibitory effect of the wetting agent can be corrected by the addition of sphingomyelin to the medium, even in protein-free media.

Comparative Effects of Tween 80 and Triton A20 on the Morphological Characteristics of Cultures H37Rv and H37Ra.—Unlike Tween 80, Triton A20 can inhibit the growth of the avirulent variants of mammalian tubercle bacilli in concentrations which exert no detectable toxic effects on the virulent forms (Tables I and III). This difference in antibacterial activity between the two types of wetting agents is paralleled by differences in their effect on the morphological aspects of the cultures growing in their presence. Only a brief statement of these findings will be presented here as the relation of morphological characteristics to virulence is to be treated more extensively in a subsequent communication.

It will be recalled that, in the absence of a wetting agent, the virulent organisms exhibit a marked tendency to adhere to one another in the direction of their long axis; this tendency results in the formation of strands of bacilli, which can be very long and at times extend over several microscopic fields, and which are several cells in thickness. In contrast to this serpentine pattern of growth, the avirulent forms exhibit either random growth or perhaps a rosette

arrangement of the cells (7). Attenuated strains, which possess, like the BCG culture in use in our laboratory, a slight degree of "invasiveness" also exhibit intermediate morphological characteristics. On the other hand, in media containing 0.1 per cent Tween 80, cultures of the virulent organisms and of the attenuated forms grow without producing the cords characteristic of growth in the plain albumin medium, and cannot be differentiated from the totally avirulent variants. A different morphological picture is obtained in media containing the same concentration of Triton A20 (0.1 per cent). Although growth of the virulent variants in Triton A20 liquid media appears fairly disperse, and their colonies in Triton A20-albumin agar are much less rugose than those developing on plain albumin agar, microscopic examination reveals that these cultures exhibit a definite serpentine pattern of growth (cord formation). It would thus appear that Triton A20 prevents the formation of the amorphous large clumps which correspond to the granular mode of growth of tubercle bacilli in general, but does not interfere with the tendency of the virulent cells to orient themselves in the typical "cords." These observations suggest that two independent factors contribute to the morphology of cultures of tubercle bacilli: one, common to both virulent and avirulent forms, is overcome by both Tween 80 and Triton A20, the other, peculiar to the virulent variants, is affected only by Tween 80. It is also possible, however, that these differences are not of a qualitative nature but may be due to the possession by the virulent variants of larger amounts of a certain hydrophobic substance, much less abundant in the avirulent forms, and which is more readily wetted by the water-dispersible esters than by Triton A20.

Whatever the ultimate significance of these findings for the understanding of the nature of the differences in cellular structure between the virulent and avirulent variants, it appears that the wetting agents Tween 80 and Triton A20 may lend themselves to the development of selective media useful for the separation of the two types of variants and for the analysis of bacterial variation and its relation to virulence.

SUMMARY

Tween 80 and Triton A20 are two water-dispersible, non-ionic, surface-active agents which favor dispersed growth of tubercle bacilli in aqueous media probably by wetting the bacterial surface.

Tween 80 is a polyoxyethylene ester of sorbitan monooleate and is liable to enzymatic hydrolysis by lipases. Triton A20 is an arylalkyl polyether of phenol which appears resistant to the known enzymes of animal tissues.

Tween 80 loses its ability to disperse cultures of tubercle bacilli in media containing serum; Triton A20 does not.

Tween 80 increases the yield of growth, probably by supplying oleic acid to the bacilli; Triton A20 does not.

In concentrations sufficient to cause dispersed growth, Tween 80 (purified by removal of unesterified fatty acid) and Triton A20 are completely innocuous for virulent tubercle bacilli. However, Triton A20 exhibits a marked toxic effect on the avirulent variants of mammalian strains; Tween 80 does not.

The two wetting agents also differ in their effects on the morphological aspects of the bacterial cultures. Whereas Triton A20 prevents the formation of large amorphous bacillary clumps, it is less effective in preventing the orientation of the virulent bacilli resulting in the formation of long bacillary strands. Tween 80 on the contrary prevents also the formation of these cords of bacilli and exerts therefore a more effective dispersing effect on cultures of virulent tubercle bacilli.

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SPECIFIC SERUM AGGLUTINATION OF ERYTHROCYTES SENSITIZED WITH EXTRACTS OF TUBERCLE BACILLI

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It is known that red blood corpuscles can adsorb various substances and thereby be rendered specifically agglutinable by serum antibody directed against the substance adsorbed (1-4). The present paper describes observations on the agglutination, by the sera of experimental animals and of tuberculous patients, of erythrocytes previously treated with aqueous extracts of tubercle bacilli or with products of their culture filtrate.

Materials and Methods

Preparation of Extracts of Tubercle Bacilli.—The H37Rv strain of human tubercle bacillus was cultivated on the surface of a liquid medium described in a previous communication (medium 7 in reference 5). After approximately 14 days of growth, the whole bacillary mass was filtered free of the culture filtrate and washed on the filter, first with distilled water, then with cold acetone. Following removal from the filter, the organisms were air-dried at room temperature in a glass container. All these operations were carried out under irradiation with ultraviolet light. The dried bacillary material was stored at room temperature or in a refrigerator.

Four grams of air-dried bacilli was suspended in 100 cc. of 88 per cent phenol (phenol liquefied, U. S. P. XIII, Mallinckrodt) and stirred by a magnetic stirrer at 35°C. for 20 hours; the suspension was centrifuged and the clear brownish supernatant fluid discarded. The sediment was resuspended in 100 cc. of fresh phenol solution and the same operation was repeated. The new sediment was twice resuspended in fresh phenol solution, stirred for only $\frac{1}{2}$ hour each time, and centrifuged. It was then washed three times with 90 cc. aliquots of cold acetone in order to remove the phenol. Finally, the phenol-acetone-treated bacillary mass was dried and stored at room temperature.

The phenol-acetone-treated, dried bacillary material (0.5 gm.) was suspended with mortar and pestle in an isotonic aqueous solution (65 cc.) containing 0.55 per cent NaCl, 0.5 per cent Na_2HPO_4 , and 20 per cent methanol. The methanol served to facilitate suspension of the bacilli as well as to prevent contamination. This suspension was stirred for 20 hours by a magnetic stirrer at 35°C. The extract was freed of most of the suspended bacillary bodies by centrifugation in an angle head centrifuge (Sorval type) at 10,000 R.P.M. for 30 minutes. The slightly opalescent extract was adjusted to a pH of 6.0, was dialyzed through ordinary sausage casing cellophane against tap water for 6 hours, and then against distilled water for 1 to 3 days. The solution in the cellophane bag was evaporated down to a volume of 20 cc. in front of a fan, removed from the bag, and centrifuged at high speed to yield an almost clear extract which was adjusted to pH 6.5 to 7.0 with dilute NaOH and made isotonic by the addition of 0.52 cc. of saturated aqueous solution of sodium chloride.

Preparation of Washed, Packed, Sheep Erythrocytes.—Sheep's blood was collected aseptically in 1.2 volumes of sterile modified Alsever's solution (6). The red cells in this mixture remain adequate for the hemagglutination test for 3 months when kept under aseptic conditions at

4°C. The mixture of blood and Alsever's solution was centrifuged and the red cells were washed three times with 6 volumes of isotonic saline solution. After the last centrifugation at 2000 R.P.M. for 20 minutes, as much of the supernatant fluid as possible was removed and the packed cells were stored at 4°C.; these cells were used within 3 days or until hemolysis was clearly evident. Fifty cc. of blood-Alsever's mixture yielded about 7 cc. of packed, washed erythrocytes.

Sensitisation of Sheep's Cells with Extract.—Approximately 0.5 cc. of packed, washed red cells was added to 10 cc. of the neutral, isotonic extract of tubercle bacilli; the cells were well suspended and placed in a water bath at 37°C. for 2 hours and frequently agitated; then the suspension was centrifuged and the clear supernatant fluid was discarded. The packed treated red cells were washed three times with 30 cc. of saline and finally suspended in 100 cc. of sterile saline (0.5 per cent "sensitized red cell suspension") to be stored at 4°C. and used within 3 days or until hemolysis was clearly evident.

Collection and Preparation of Serum for Absorption.—Blood was collected and allowed to clot, and the clot allowed to retract; serum was removed and, for preservation, when desired, merthiolate was added in a final concentration of 0.01 per cent. To 1 cc. of serum was added 1 cc. of saline and this mixture was heated at 56°C. for 30 minutes in order to inactivate complement.

Absorption of Serum with Untreated Red Cells.—The following operations were designed to remove from the sera, antibodies unrelated to tuberculous infection which would agglutinate untreated red cells by the hemagglutination technique to be described.

In 2 cc. of 1:2 dilution of heated serum 0.2 cc. of packed, washed, untreated sheep's cells was suspended, and the suspension was allowed to stand at room temperature for at least 20 minutes, during which it was frequently shaken. It was then centrifuged to sediment the cells and the supernatant fluid was removed and treated in the same way with another 0.2 cc. of packed untreated cells. The final supernatant fluid was used in the hemagglutination tests as representing a 1:2 dilution of absorbed serum.

A 0.5 per cent suspension of untreated red cells, to be used as control antigen in the testing of all sera, was prepared by suspending 0.1 cc. of untreated, washed, packed sheep cells in 20 cc. of isotonic sterile saline. This suspension was stored in the refrigerator and used no longer than 3 days after its preparation.

Performance of Hemagglutination Test.—Twofold serial dilutions of the absorbed serum to be tested were made in saline in tubes of 12 mm. internal diameter. To tubes containing 0.4 cc. of serum dilution there was added 0.4 cc. of 0.5 per cent suspension of treated erythrocytes. Two control tubes were found to be necessary: one containing 0.4 cc. of saline and 0.4 cc. of 0.5 per cent suspension of treated cells, and the other containing 0.4 cc. of 1:2 dilution of absorbed serum and 0.4 cc. of 0.5 per cent suspension of untreated erythrocytes.

Incubation at 37°C. for 2 hours, followed by a preliminary observation, a vigorous shaking, and further incubation at room temperature overnight, was found to allow satisfactory reading of the degree of agglutination. The results were read and recorded as in any red cell agglutination test.

Agglutination of the treated cells in saline has usually been present to an insignificant extent and this has served as a guide in the judging of clear cut agglutination in an actively agglutinating serum. Agglutination of untreated red cells by the absorbed serum indicates that the serum has been incompletely absorbed and renders the test invalid.

Technique of Inhibition of Specific Hemagglutination by Solutions Containing Sensitising Material.—An active serum was selected which agglutinated treated red cells at high titre, and a serum dilution near the end-point of agglutination was carefully determined which consistently gave definite, though not strong, agglutination of treated red cells. Serum in this dilution was added in 0.4 cc. amounts to a series of tubes containing 0.4 cc. of serial dilu-

tions of the substance to be tested for its ability to inhibit the specific agglutination. The tubes were incubated in a water bath at 37°C. for 45 minutes. To each tube was then added 0.4 cc. of a 0.5 per cent suspension of treated red cells. Incubation and the reading of results were carried out as in the case of the above described hemagglutination test. Appropriate controls containing untreated red cells with the undiluted solution to be tested, treated cells with saline, and treated cells with serum, respectively, were included in such tests.

EXPERIMENTAL RESULTS

Study of Sera.—Table I presents the results obtained in the examination of various active sera.

TABLE I
Agglutination of Sensitized Red Cells by the Sera of Rabbits Injected with BCG

Dilutions of sera in saline	Immune rabbit sera						Normal rabbit serum
	1 (742)	2 (744)	3 (745)	4 (734)	5 (735)	6 (736)	
1:10	++++	++++	++++	++++	++++	++++	—
1:20	++++	++++	++++	++++	++++	++++	—
1:40	++++	++++	++	++++	++++	++++	—
1:80	++++	++++	+	++++	++++	++++	—
1:160	+++	+++	—	++++	++++	++++	—
1:320	+	+	—	++++	++++	++++	—
1:640	—	—	—	+++	+++	++	—
1:1280	—	—	—	++	+	+	—
1:2560	—	—	—	—	—	—	—
1:10 with untreated red cells	—	—	—	—	—	—	—

Sera 1, 2, and 3 were drawn from rabbits which had received 8 weeks previously one single intravenous injection of 0.2 mg. of a living culture of a strain of BCG identified as BCG 317 (7). Sera 4, 5, and 6 were drawn from rabbits injected according to a different schedule: the animals were injected by the intravenous route with 0.2 mg. of a living culture of the same strain of BCG and were allowed to rest for 5 weeks; then three weekly injections of 0.02 mg. of the same culture in 1 cc. of saline were made into the left heart in order to distribute the bacilli into parts of the body other than the lung.

The data presented show that the titres of antibody against the treated red cells were consistently and significantly higher in the rabbits repeatedly injected with tubercle bacilli.

In Table II are recorded the results obtained with the sera of some patients with active pulmonary tuberculosis. No correlation has yet been attempted between the degree of activity of the disease and the titre of the serum in the hemagglutination test, but it is evident that all the patients tested had titres

of 1:8 or higher; that is to say, they possessed antibodies capable of agglutinating the sensitized red cells. One additional serum from a patient with miliary tuberculosis, made available to us by Dr. Walsh McDermott of the New York Hospital, had a titre of 1:256.

Some evidence has been obtained for the specificity of the hemagglutination reaction by testing red cells treated with the extract of tubercle bacilli against the sera of experimental animals which had been immunized with other micro-organisms:¹ *Pneumococcus* Type I, *Pneumococcus* Type III, *Pneumococcus* Type XIV, *Friedländer* bacilli types B and C, and *Flexner* dysentery bacilli types X and Y. There were weak cross-reactions with *Friedländer* antisera which gave doubtful agglutination at 1:2 dilutions of serum; these reactions can be considered insignificant. There were also weak reactions with high

TABLE II
Agglutination of Sensitized Red Cells by the Sera of Tuberculous Patients

Dilutions of sera in saline	Human sera						Saline control
	1	2	3	4	5	6	
1:2	+++	++++	+++	++++	++++	++++	—
1:4	++	++++	++	++++	++++	++++	—
1:8	+	++++	+	++++	+++	++++	—
1:16	—	++	±	+++	+++	++	—
1:32	—	±	—	++	++	+	—
1:64	—	—	—	+	±	—	—
1:2 with untreated red cells	—	—	—	—	—	—	—

titled *Pneumococcus* Type XIV antisera, both from the horse and from the rabbit; these were not marked, and appeared only in the dilution below 1:32.

Table III gives further evidence for the specificity of the reaction. Serum 1 was a pool of sera from 10 to 20 individuals all giving strongly positive Wassermann reactions for syphilis;² it is probable that many of these individuals were tuberculin-positive, but there was no evidence that any of them had active tuberculous disease. The pooled serum was also investigated for its ability, at a dilution of 1:4, to inhibit specific hemagglutination by a selected active serum, but there was no evidence of such inhibitory activity. Sera 2, 3, 4, and 5 were from patients with or convalescing from acute streptococcal infections.³

¹ Kindly supplied for our use by Dr. Walther Goebel and Dr. Frank Horsfall of the Rockefeller Institute.

² This pool was generously supplied by Dr. Widlock of the Serology Laboratories of the New York City Department of Health.

³ Made available through the kindness of Dr. Sidney Rothbard of the Hospital of the Rockefeller Institute.

Sera 6, 7, 8, 9, and 10 were from tuberculin-negative student nurses at the New York Hospital.⁴ Serum 11 was from a tuberculin-positive individual who had been working for many months with living tubercle bacilli but had no evidence of active disease. As will be noted from the results presented in Table III, none of these sera gave titres higher than 1:4 in the hemagglutination test.

Study of Antigen.—Material from the tubercle bacillus which sensitizes red cells to agglutination in tuberculous antisera has been demonstrated to be present in at least one preparation of old tuberculin. Thus, one sample of Gilliland O. T. (Wyeth), which had been steamed for many hours during preparation, was observed to be as effective in sensitizing sheep erythrocytes as the extract described here. One ml. of this deglycerinated O. T.⁵ was capable

TABLE III
Agglutination of Sensitized Red Cells by the Sera of Non-Tuberculous Individuals

Dilutions of sera in saline	Human sera											Saline control
	1	2	3	4	5	6	7	8	9	10	11	
1:2	±	—	—	—	—	++	—	—	—	++	+	—
1:4	—	—	—	—	—	±	—	—	—	+	—	—
1:8	—	—	—	—	—	—	—	—	—	±	—	—
1:16	—	—	—	—	—	—	—	—	—	—	—	—
1:32	—	—	—	—	—	—	—	—	—	—	—	—
1:2 with untreated red cells	—	—	—	—	—	—	—	—	—	—	—	—

of sensitizing completely 0.025 cc. of washed packed sheep erythrocytes to such an extent that they were agglutinated in the same way and to the same titre in an anti-BCG hyperimmune serum (No. 734) as were the cells of the same lot sensitized by the above described extract of tubercle bacilli. This fact shows that at least one substance responsible for sensitization is heat-stable and is present in the culture filtrate of cultures of mammalian tubercle bacilli.

Additional experiments were performed in an attempt to determine whether active material was present in the carbohydrate or in the protein fraction of the bacillary extracts and culture filtrates. Serial dilutions of these fractions were made in saline and were tested for their relative ability to inhibit specific hemagglutination of red cells sensitized by the extract of H37Rv. Table IV reveals the results of these experiments. Extract fraction 1 was the crude aqueous extract described in this paper. Extract fraction 2 was an aliquot of

⁴ Made available through the kindness of Dr. F. Lansdown and Dr. C. Muschenheim of the New York Hospital.

⁵ Available through the courtesy of Dr. Merrill Chase of the Rockefeller Institute.

the same extract from which most of the protein had been removed by acidification with dilute HCl and repeated shaking with chloroform and isoamyl alcohol (8) until there was very little material at the chloroform-water interphase after centrifugation. It is evident that this procedure failed to decrease significantly the ability of the extract to inhibit specific hemagglutination even by a low dilution of antiserum.

TABLE IV³

Inhibition of Specific Hemagglutination by Extracts and Fractions of Tubercle Bacilli

Dilutions of extracts in saline	Extracts and fractions							
	1*	2*	1†	2†	1§	2§	3§	4§
Undiluted	—	—	—	—	—	—	—	—
1:2	—	—	—	—	—	—	—	—
1:4	—	—	—	—	—	—	—	—
1:8	—	—	—	—	—	—	+	—
1:16	—	—	—	—	—	—	++	—
1:32	—	+	—	—	—	—	++	±
1:64	++	+++	—	—	—	—	++	+
1:128	+++	+++	—	—	—	—	++	++
1:256			—	—	—	—	++	++
1:512			—	—	—	—	++	++
1:1024			—	—	+	+	++	++
1:2048			—	—	++	++	++	++
1:4096			±	+				
1:8192			++	++				
Saline control	+++	+++	++	++	++	++	++	++

* Tested against 1:10 dilution of BCG rabbit antiserum 735, absorbed with untreated red cells.

† Tested against 1:320 dilution of BCG rabbit antiserum 735, absorbed with untreated red cells.

§ Tested against 1:320 dilution of BCG rabbit antiserum 734, absorbed with untreated red cells.

Unheated culture filtrate fractions 3 and 4, made available for our use by Dr. Janet McCarter Woolley, had been defined chemically as follows. Fraction 3 was known to contain only very little polysaccharide and approximately 20γ/0.4 cc. of tuberculo protein as determined by non-nucleic acid nitrogen content. Fraction 4, on the contrary, contained approximately 20γ/0.4 cc. of tuberculo polysaccharide and no more than one part of tuberculo protein to 19 parts of polysaccharide. It is evident that material active in the hemagglutination test was in the polysaccharide fraction and that amounts as small as 0.16γ/0.4 cc. of solution could be detected by the technique of specific inhibition

of hemagglutination. It is clear, also, that it is possible by these techniques to standardize a solution of any extract or product of the culture filtrates of tubercle bacilli, with respect to activity in sensitizing sheep erythrocytes for the specific hemagglutination test.

The Use of an Avirulent Culture of Tubercle Bacilli for the Preparation of Sensitized Sheep Erythrocytes.—The avirulent variant, H37Ra (8), of the virulent strain, H37Rv, has been investigated for its ability to yield material capable of sensitizing sheep erythrocytes and to inhibit the specific hemagglutination of erythrocytes treated with the extract of H37Rv. No significant differences of a qualitative nature have, thus far, been detected in these respects between the two variant strains by the use of the high titred experimental animal sera or of sera of tuberculous patients.

DISCUSSION

The observations described show that at least one heat-stable component present in a polysaccharide fraction of the tubercle bacillus, can be adsorbed onto sheep erythrocytes, rendering them specifically agglutinable by antibody directed against the adsorbed material. The antibody responsible for this hemagglutination test circulates in the blood of immunized animals and of human beings with active tuberculosis. The test exhibits a high degree of specificity and, in particular, does not give rise to any cross-reaction with Wassermann-positive sera as sometimes occurs in the case of the complement fixation reactions in tuberculosis (9).

The specific hemagglutination reaction can be inhibited by adding the soluble reactive antigen to the serum before introducing the sensitized red cells into the system. Under these circumstances the soluble antigen, if present in sufficient amount, combines with its corresponding antibody and prevents it from agglutinating the sensitized erythrocytes. Thus this inhibition test, when utilized together with the agglutination test proper, permits the detection and quantitation of very small amounts of the sensitizing antigen, as repeated observations have shown. It will be interesting to test whether this technique permits the detection of specific antigen circulating *in vivo*.

SUMMARY

A hemagglutination reaction has been described between sheep erythrocytes treated with a component of a polysaccharide fraction of mammalian tubercle bacilli and the sera of experimental animals or of tuberculous patients.

Evidence has been presented for the specificity of this reaction. A modification of the test, involving an inhibition reaction, has been developed for the detection and quantitation of minute amounts of the material responsible for the hemagglutination reaction.

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THE QUANTITATIVE SEPARATION OF PURINES, PYRIMIDINES, AND NUCLEOSIDES BY PAPER CHROMATOGRAPHY

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The separation of amino acid mixtures by migration with organic solvents in filter paper has been successfully accomplished by many workers since it was first described by Consden, Gordon, and Martin (1). Each amino acid travels in a more or less well defined spot in the body of uniformly migrating solvent and can be visualized in the dried paper as a local spot giving a color reaction with ninhydrin. The present paper reports the separation of the purines and pyrimidines contained in nucleic acids, and several related compounds, by the movement of a boundary of *n*-butyl alcohol along paper strips. Vischer and Chargaff (2) have described the principal steps of a procedure for separating the two bases, guanine and adenine, from nucleic acid in a moving body of a quinoline-collidine mixture. These purines were located in the paper by precipitation of mercury sulfide after formation of the insoluble mercury salts and washing in dilute acid. After complete removal of the quinoline and collidine it was possible to identify the guanine and adenine by their absorption maxima in the ultraviolet range.

Advantageous features of the method described below are as follows: (a) The five bases, cytosine, thymine, uracil, adenine, and guanine, can in microgram quantities be completely separated from one another. (b) The separated substances are detected by ultraviolet spectrophotometric examination of solutions prepared by soaking excised portions of the paper in water. (c) An organic solvent is used which does not interfere with ultraviolet spectrophotometry. (d) Recovery of the individual constituents is essentially quantitative, with an error of 10 per cent or less. (e) Spectrophotometric standards of purity are provided which make it possible to identify each component and (f) to determine with considerable accuracy the composition of binary mixtures which may result from a migration not carried far enough to give altogether complete separations. (g) 1 mg. or less of a nucleic acid can be examined for the rate of liberation of its nitrogenous bases during partial enzymatic or acid hydrolysis. (h) Some simple derivatives such as nucleosides can be purified and identified in this procedure. (i) Less soluble substances, or dilute solutions, are used by evaporation of moderately large volumes of solution upon a small spot in the paper, locally heated.

Certain aspects of the method remain unsatisfactory or disadvantageous: (a) Guanine does not migrate appreciably in butyl alcohol and so it remains with unhydrolyzed nucleic acid, nucleotides, or other alcohol-insoluble substances in the initial spot. Without use of a second solvent, guanine can therefore be isolated in pure form only from certain simple mixtures or completely hydrolyzed material. (b) Phenylalanine (and possibly tryptophan, but not tyrosine) may, if present, appear on the paper in the region occupied by cytosine. Its presence can be recognized through the ninhydrin color reaction or through spectrophotometric study of the fractions. While certain synthetic phenols and aromatic acids would likewise interfere, probably very few natural products will be found to do so. (c) As in other paper chromatography, large quantities of the substances or even of certain foreign materials often tend to make the separation less sharp. (d) Variations in water content of the paper, or other ill defined factors, make it difficult to predict exactly the position that will be occupied by isolated compounds; relative positions and relative rates of flow, on the other hand, are constantly reproducible.

Some of the disadvantages mentioned may eventually be overcome through use of successive solvents or a different initial solvent. The method as described promises to be useful in the quantitative study of the composition of nucleic acids, their constituents and derivatives, and the manner of their enzymatic or hydrolytic degradation.

EXPERIMENTAL

Apparatus—The majority of the chromatograms were developed in a Pyrex glass cylinder surmounted by a flanged aluminum ring closely fitting the cylinder and serving as the support for brass arms holding horizontally placed cylindrical glass troughs and glass rod separators, about 140 mm. in length. A glass plate is placed upon the plane upper surface of the aluminum ring to give a closed system. Early experiments were successfully carried out in an ordinary glass and metal aquarium resting upon one end and closed at the side with a glass plate. In this chamber a wood and metal ring stand supported a rectangular, glass staining dish cover, which served as the trough. The metal used was not observed to cause any disturbances in either of the chambers; wood, however, becomes swollen and saturated with solvent vapor, and should not be used in systems with more than one organic solvent.

Volumes of solution larger than 0.017 cc. were placed upon a small area of the paper, locally heated by curving the strip over a horizontal glass tube, 10 mm. in diameter, joined between a flask of boiling water and a reflux condenser. The fluid samples are delivered from a calibrated capillary pipette (3) by touching it to the paper from time to time as drying occurs.

There has been no indication that the presumptive local drying of the paper has influenced the resolution of either pyrimidines, purines, or amino acids.

Solutions were examined in 3 cc. quantities in the model DU Beckman photoelectric quartz spectrophotometer.

Materials—The paper used was Whatman No. 1, as recommended for amino acid determinations (1). Aqueous extracts of this paper have only very low absorption in the ultraviolet, as discussed below. *n*-Butanol was used as the organic solvent in all of the work reported here. The bottom of the chamber was covered with equal portions of butanol and water and the aqueous phase was brought to about 2.5 per cent concentration of gaseous ammonia by addition of concentrated ammonia. The troughs contained butanol saturated with water at the prevailing room temperature, without added ammonia.

Two lots of each of the principal bases were used: from Hoffmann-La Roche, Inc. (Basel), adenine, guanine, and uracil, kindly made available by Dr. A. Claude; from the Schwarz Laboratories (New York), adenine sulfate, guanine, thymine, and yeast nucleic acid; from the Eastman Kodak Company (Rochester), uracil and yeast nucleic acid; from the Dougherty Chemicals (Richmond Hill, New York), cytosine. In addition, crystalline samples of thymine, cytosine, xanthine, hypoxanthine, adenosine, cytidine, guanosine, and thymidine, prepared in the laboratory of the late Dr. P. A. Levene, were employed. Desoxyribonucleic acid was prepared from calf thymus according to the method of Mirsky (4).

Weighed amounts of the pure bases were dissolved in water, neutralized to about pH 7 if necessary, and the concentration accurately determined by Kjeldahl determination of nitrogen. From these solutions the standard curves for pure bases were obtained and the mixtures submitted to separation were prepared. The nucleosides were not available in sufficient quantity for nitrogen analysis; accordingly the relative absorption data are more dependable than the absolute for these substances.

All calculations based upon absolute weight throughout this paper refer to the anhydrous free bases.

Nucleic acids were hydrolyzed in aqueous hydrochloric acid for 2 hours at 120° in the autoclave. The acid was later removed by evaporation to dryness *in vacuo*, and the hydrolysate, dissolved in a small volume of water, was neutralized with sodium or ammonium hydroxide.

Preparation of Paper Chromatogram—The solutions to be investigated are deposited upon spots or bands distributed along a pencilled "starting line" drawn transversely about 50 mm. from one end of the paper strip. The spots are narrow (10 mm. or less) in the direction of flow, but along the starting line are spread for a distance such that each mm. of length carries approximately 1 γ (and preferably not more than 5 γ) of the individual

bases. Most of the work described here was done on paper strips 125×460 mm., bearing three or four initial spots approximately 10×20 to 25 mm. in size, arranged at least 10 mm. apart. The solutions are applied in the manner indicated above to these spots outlined in pencil. Such spots can carry 20 to 100 γ of individual bases or the degradation products of 1 mg. of nucleic acid.

The end of the paper strip is inserted into a glass trough and weighted by a large glass rod so that the starting line, which is near this end, is just at the point where the paper curves over the glass rod separators and projects downward. After the strips are in place and the bottom of the chamber is provided with butanol-water as mentioned, *n*-butanol saturated with water at room temperature is added to the trough and the chamber is closed. A jacket of corrugated paper is placed about the cylinder to protect it from draughts and exposure to radiators or windows, which can have disastrous effects on an unprotected system.

After 16 to 24 hours at about 25° , the position of the butyl alcohol front is marked, and the strip is removed and hung upside down to dry. The individual "lanes" can be cut apart at this time for separate treatment with ninhydrin, etc., or for separate cutting of strip segments.

Transverse segments are now cut from each lane on a trimming board or with scissors at successive chosen intervals, such as every 10 mm. The precision of the method may often justify making segments as narrow as 5 mm., and in vacant areas segments of 20 or 30 mm. width are convenient. Each excised piece is placed in a clean test-tube and then soaked 1 hour or more in 3.5 cc. of distilled water. In this work the paper may be touched with the hands, but is preferably subjected to a minimum of such exposure.

The absorption of the eluted solutions at $260 m\mu$ is determined in the spectrophotometer. A graph or table of this absorption correlated with the distance travelled from the starting line (to the mid-point of the segment) reveals a series of absorption peaks corresponding to the constituents which have been separated from the original mixture. Figs. 1 and 2 and Table I present typical data. It will be seen that the presence of several substances in each mixture is clearly indicated.

The identity of the separated substances is ascertained by more detailed spectrophotometric examination of the various eluted solutions. In the work to be described below, 3 cc. of these solutions were placed in the quartz cuvettes and examined as desired, either in this approximately neutral state or after being made 0.1 *N* in alkali or acid. For the latter purposes, 0.06 cc. (in routine work, 1 drop) of 5 *N* sodium hydroxide was added, followed when desired by 0.05 cc. (or 1 drop) of 12 *N* (or concentrated) hydrochloric acid.

In what follows the procedures are grouped according to four aims to be achieved: (a) rigorous test of the method, or of new solvents, etc., by study

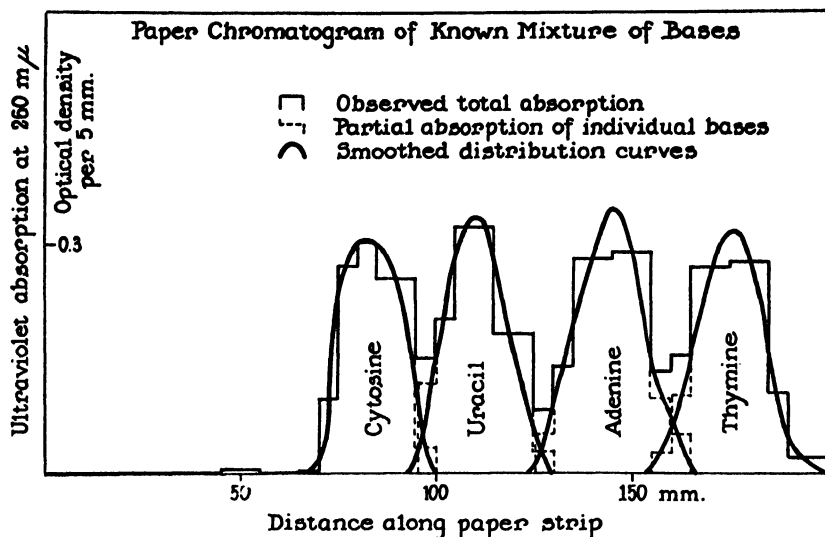


FIG. 1. Distribution of known bases in paper chromatogram

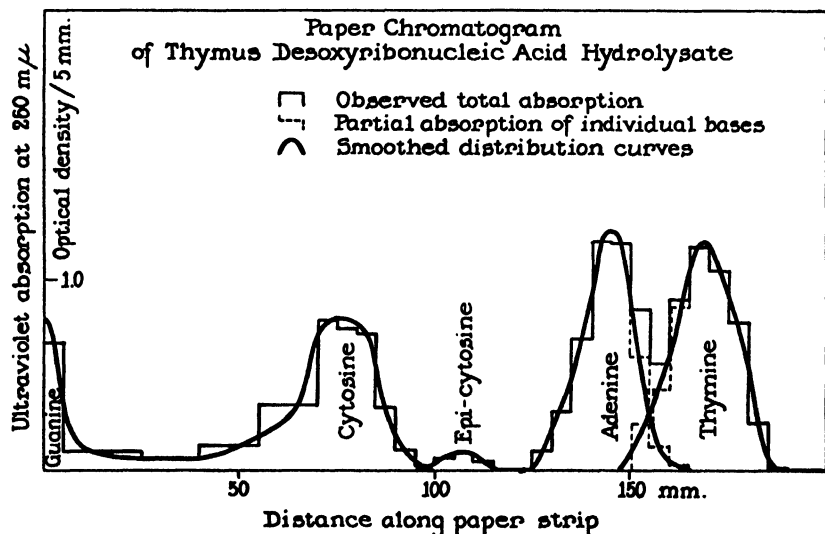


FIG. 2. Distribution of bases from thymus deoxyribonucleic acid hydrolyzed 2 hours at 120° in 2.4 N hydrochloric acid.

of identity and purity of solutions obtained at or near the positions of peak absorption, (b) routine identification of substances isolated near the peaks, (c) routine procedure for determination of quantitative composition of

those pure solutions or binary mixtures which may be obtained at the low points between the absorption peaks, and (d) complete quantitative analysis of mixtures of bases.

Identification of Isolated Major Constituents—Those eluted solutions having high absorption values at 260 $m\mu$ may be further studied to determine their composition. In order to demonstrate in this investigation that pure pyrimidines and purines may be recovered from hydrolysates of nucleic

TABLE I
Position of Pyrimidines and Purines in Butanol-Paper Chromatograms

Substance	Range of 80 per cent distribution in paper				Average R_F^* values, all experiments (300-350 mm.)
	Known Mixture I	Known Mixture II	Yeast nucleic acid hydrolysate	Thymus nucleic acid hydrolysate	
	mm.	mm.	mm.	mm.	
Solvent front	377	310	360	360	
Thymine	205-223	167-184		172-188	0.54
Adenine	176-195	138-159	143-163	141-161	0.45
Uracil	125-144	107-123	109-132		0.35
Cytosine	98-115	81- 96	82- 97	66- 97	0.26
Guanine	0	0	0	0	0.0
		Known Mixture IV	Known Mixture V	Known Mixture VI	
Solvent front	377	340	340	351	
Thymine desoxyriboside			160-183		0.51
Adenosine	118-139	96-113			0.33
Cytidine	56- 77		35- 61		0.16
Guanosine	35- 50	22- 41			0.10
Guanine desoxyriboside				65- 84	0.21
Xanthine					0.01
Hypoxanthine					0.18

* R_F = mm. traveled by the base per mm. traveled by the solvent past the starting line; maximum variation, ± 0.03 . Quantitative range of distribution, determined as described in a later section.

acids and from known mixtures, the absorption curves from 220 to 310 $m\mu$ were determined at intervals of 5 $m\mu$ in the Beckman spectrophotometer. Standard curves for authentic samples of pure bases and curves from typical samples recovered on paper strips are given in Figs. 3 to 5. The close correspondence of the data for authentic and recovered bases indicates that the substances are isolated essentially free of other ultraviolet-absorbing material. The case of guanine will be discussed more fully below.

In identification and determination of purity of bases, it has proved useful

to treat the quantitative data on the basis of ratios to a peak optical density arbitrarily taken as 1.0 at the absorption maximum. The maximum, normally around $260\text{ m}\mu$, is determined within $5\text{ m}\mu$ and all optical density values are divided by the value found at this wave-length. Expression of results in this form facilitates comparison of curves obtained with widely differing concentrations. With the spectrophotometer used the curves are reliable enough for identification of the common purines and pyrimidines

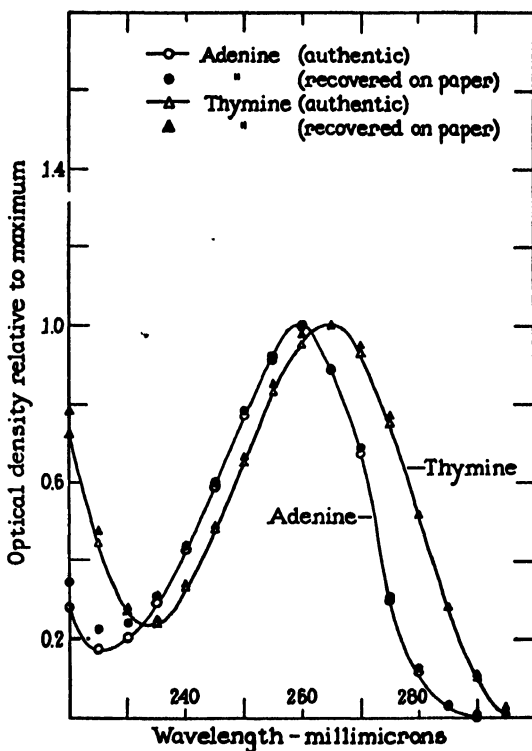


FIG. 3. Relative absorption curves of adenine and thymine in neutral solution

whenever the peak absorption is from 0.15 to 1.50 optical density units. With lower total absorption it becomes more important to make the blank corrections mentioned below.

The relative absorption data for the five bases in neutral, alkaline, and acid solutions are collected in Table II, together with similar standards for certain related compounds. These standard values are in approximate agreement with those estimated as accurately as may be done from the curves available in the literature (5-7). The absolute absorption, N_{260} , of a unit concentration is also given, and is used in the calculation of absolute

weight of a base from absorption measurements. The ratios of the maxima in alkaline and acid solution to the neutral maximum, $\text{max.}/N_{\text{max.}}$, are also given and indicate the shifts in magnitude of absorption at the different acidities. These shifts are in some cases highly characteristic, and do not appear from the literature to be well known.

Not only is the correspondence between the absorption of authentic and recovered bases in neutral solution regularly as satisfactory as indicated in Figs. 3 to 5, but equally good agreement is found in data from alkaline or

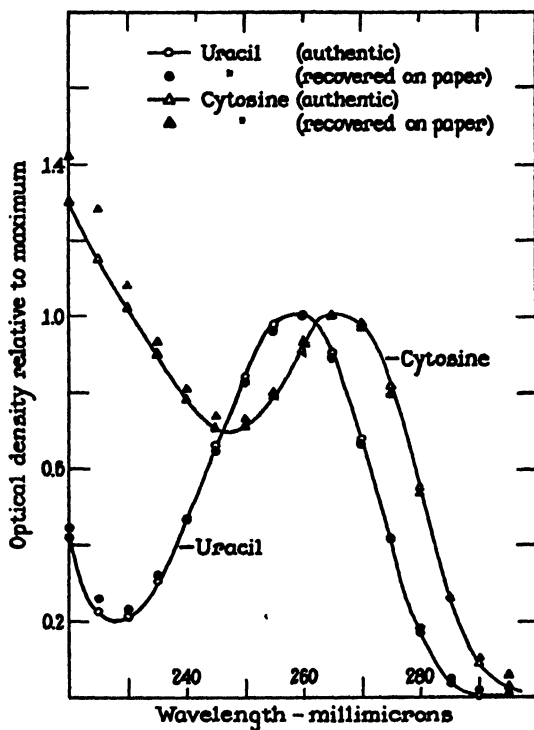


FIG. 4. Relative absorption curves of uracil and cytosine in neutral solution

acid solutions (not shown here). The recovered guanine alone is not highly pure, although it is readily identifiable. Undoubtedly the difficulty arises in part from the immobility of guanine in a flowing current of butyl alcohol, such that it will tend to remain in the same fraction with unhydrolyzed nucleotides or charred decomposition products which may be present in small amounts in hydrolysates. In any case, since guanine is extremely insoluble in water, only small amounts of it will be encountered; simple filtration or centrifugation suffices to recover a large part of the guanine present in a neutralized hydrolysate.

In precise work it is desirable to correct absorption measurements for blank errors such as cuvette absorption and the absorption of an eluate from a blank portion of the solvent-treated paper. The latter correction varies for the paper mentioned from 0.01 (optical density units) at 280 $m\mu$ to perhaps 0.03 at 220 $m\mu$ for a 10 mm. segment cut from a 40 mm. lane and may therefore be neglected in usual routine work. It is apparently due to substances having low terminal absorption in the ultraviolet range and is not materially affected by the passage of a butyl alcohol front through

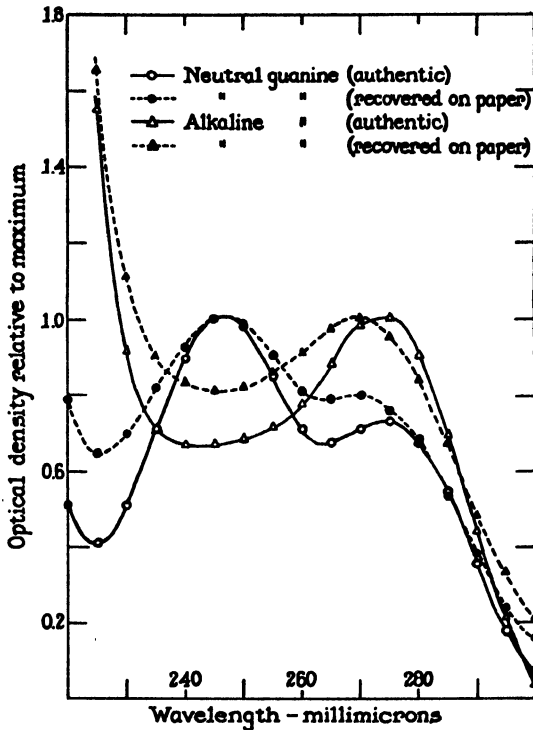


FIG. 5. Relative absorption curves of guanine in neutral and alkaline solution

the paper. Previous washing of the paper with water reduces it to some extent, but is not recommended since uneven drying appears to produce local changes in the texture and capillary properties of the paper.

The blank errors of the sort just mentioned as well as fluctuations of the pH tend to make reproducibility of the standard curves somewhat less good in the range 220 to 240 $m\mu$, even with authentic samples; therefore, undue weight is not placed upon comparisons made in this portion of the spectrum.

In Fig. 2 a minor constituent designated "epicytosine" is indicated, having a migration rate somewhat greater than that of cytosine. This small peak

TABLE II
Standard Ultraviolet Absorption Data on Purines and Pyrimidines

Wave-length, mμ	Thymine			Adenine			Uracil		
	OH	N	H	OH	N	H	OH	N	H
310	0.25	0	0	0	0	0	0.03	0	0
300	0.77	0	0	0	0	0	0.32	0	0
295	0.94	0.02	0.025	0.005	0	0	0.63	0	0
290	1.00	0.10	0.10	0.015	0	0.03	0.885	0	0.01
285	0.985	0.275	0.275	0.12	0.03	0.15	1.00	0.04	0.05
280	0.96	0.51	0.51	0.48	0.12	0.37	0.99	0.17	0.185
275	0.92	0.74	0.75	0.77	0.30	0.60	0.93	0.42	0.42
270	0.87	0.92	0.925	1.00	0.675	0.85	0.85	0.675	0.68
265	0.80	1.00	1.00	0.995	0.88	0.98	0.77	0.90	0.90
260	0.70	0.955	0.955	0.87	1.00	1.00	0.68	1.00	1.00
255	0.575	0.835	0.835	0.685	0.915	0.935	0.58	0.97	0.98
250	0.465	0.66	0.665	0.515	0.765	0.765	0.475	0.84	0.845
245	0.42	0.485	0.49	0.39	0.59	0.59	0.39	0.66	0.665
240	0.475	0.335	0.345	0.31	0.43	0.42	0.38	0.465	0.48
235	0.69	0.245	0.255	0.31	0.295	0.285	0.49	0.305	0.32
230	1.07	0.27	0.285	0.52	0.21	0.215	0.74	0.21	0.23
225	1.55	0.445	0.46	1.02	0.18	0.24	1.12	0.23	0.24
220	2.3	0.72	0.735	1.6	0.29	0.355	1.8	0.42	0.42
Max./N ₂₀₀	0.67	(1.0) 59	0.985	0.89	(1.0) 105	0.96	0.745	(1.0) 72	1.00
	Cytosine			Guanine			Thymine desoxyriboside		
	OH	N	H	OH	N	H	OH	N	H
310	0.005	0	0	0	0	0.01	0	0	0
300	0.13	0.005	0.045	0.04	0.07	0.08	0.02	0.01	0.02
295	0.385	0.025	0.20	0.215	0.18	0.18			0
290	0.68	0.085	0.475	0.44	0.35	0.32	0.145	0.19	0.20
285	0.895	0.26	0.73	0.70	0.54	0.44	0.345	0.40	0.395
280	1.00	0.555	0.96	0.90	0.7	0.54	0.60	0.64	0.64
275	0.94	0.82	1.00	1.00	0.75	0.60	0.83	0.855	0.85
270	0.79	0.98	0.95	0.97	0.7	0.62	0.97	0.975	0.975
265	0.62	1.00	0.805	0.875	0.68	0.65	1.00	1.00	1.00
260	0.47	0.93	0.62	0.775	0.71	0.73	0.925	0.92	0.915
255	0.36	0.80	0.45	0.72	0.845	0.90	0.80	0.775	0.775
250	0.31	0.72	0.30	0.68	0.975	1.00	0.685	0.60	0.60
245	0.35	0.72	0.19	0.67	1.00	0.99	0.645	0.435	0.44
240	0.525	0.80	0.135	0.67	0.895	0.86	0.70	0.305	0.315
235	0.795	0.93	0.145	0.71	0.71	0.68	0.855	0.24	0.25
230	1.06	1.05	0.23	0.9	0.52	0.52	1.09	0.29	0.305
225	1.25	1.2	0.425	1.6	0.41	0.44	1.3	0.46	0.48
220	1.6	1.3	0.69		0.51	0.51	1.8	0.68	0.70
Max./N ₂₀₀	1.10	(1.0) 53	1.60	0.87	(1.0) 40	1.18	0.775	(1.0) 36	1.00

TABLE II—*Concluded*

Wave-length, m μ	Guanine deoxyriboside			Guanine riboside			Cytosine riboside		
	OH	N	H	OH	N	H	OH	N	H
310	0	0	0.01	0	0	0.02	0	0	0.01
300	0	0.01	0.135	0.01	0.02	0.145	0.04	0.07	0.21
295	0.02	0.075	0.29						
290	0.09	0.22	0.45	0.095	0.15	0.455	0.31	0.41	0.745
285	0.305	0.42	0.59	0.305	0.43	0.585	0.59	0.675	0.935
280	0.60	0.58	0.665	0.595	0.575	0.655	0.825	0.88	1.00
275	0.845	0.665	0.69	0.835	0.665	0.685	0.975	1.00	0.96
270	0.97	0.715	0.71	0.96	0.71	0.72	1.00	0.98	0.84
265	1.00	0.75	0.795	1.00	0.75	0.82	0.93	0.89	0.67
260	0.99	0.86	0.935	0.99	0.86	0.965	0.81	0.765	0.495
255	0.96	1.00	1.00	0.965	0.995	1.00	0.725	0.655	0.34
250	0.86	0.995	0.945	0.87	1.00	0.92	0.705	0.60	0.225
245	0.71	0.895	0.785	0.70	0.895	0.74	0.74	0.60	0.155
240	0.54	0.695	0.585	0.55	0.68	0.54	0.81	0.625	0.14
235	0.42	0.475	0.40	0.44	0.475	0.36	0.88	0.675	0.185
230	0.40	0.30	0.26	0.43	0.31	0.25	0.925	0.75	0.31
225	0.61	0.205	0.24	0.66	0.22	0.27	0.99	0.815	0.50
220	1.4	0.21	0.38	1.6	0.25	0.45	1.3	0.91	0.68
Max./N ₂₆₀ max.	0.83	(1.0)	0.885	0.85	(1.0)	0.905	0.95	(1.0)	1.36
N ₂₆₀		44			41			30	
	Adenine riboside			Xanthine			Hypoxanthine		
310				0.06	0	0	0	0	0
300	0	0	0	0.41	0.01	0	0.01	0.01	0.01
295				0.67	0.04	0	0.04	0.03	0.03
290	0	0.01	0.05	0.89	0.12	0.04	0.08	0.06	0.06
285				1.00	0.31	0.16	0.15	0.12	0.09
280	0.16	0.165	0.23	0.98	0.59	0.39	0.31	0.18	0.14
275	0.39	0.38	0.42	0.86	0.86	0.66	0.60	0.29	0.20
270				0.70	1.00	0.91	0.84	0.43	0.32
265	0.92	0.90	0.89	0.54	0.98	1.00	1.00	0.58	0.50
260	1.00	1.00	0.995	0.43	0.86	0.97	1.00	0.75	0.70
255	0.94	0.95	1.00	0.42	0.68	0.82	0.91	0.91	0.90
250	0.775	0.805	0.84	0.48	0.53	0.64	0.75	1.00	1.00
245	0.57	0.60	0.635	0.53	0.41	0.48	0.60	0.95	0.98
240	0.40	0.42	0.44	0.57	0.36	0.41	0.46	0.79	0.86
235	0.275	0.27	0.29	0.61	0.37	0.46	0.40	0.59	0.66
230	0.24	0.18	0.23	0.78	0.39	0.51	0.48	0.40	0.46
225	0.34	0.17	0.31	1.4	0.42	0.52	0.90	0.29	0.30
220	0.8	0.37	0.58	2.8		0.52	1.8		0.23
Max./N ₂₆₀ max.	1.00	(1.0)	0.95	1.00	(1.0)	1.02	1.00	(1.0)	1.06
N ₂₆₀		54			44 Ca.			47 Ca.	

OH, N, and H refer to alkaline, neutral, and acid solutions, respectively; the absorption values are the ratios to a maximum taken as 1.00; max./N₂₆₀max. relates these maxima to the neutral maximum; N₂₆₀ at 1 mg. per cc. is the neutral absorption at 260 m μ of an absolute standard.

has been observed repeatedly in the chromatographic patterns from acid hydrolysates of a preparation of calf thymus deoxyribonucleic acid. The absorption characteristics of this material resemble those of cytosine with respect to shift in acid, alkali, etc.; nevertheless, the fraction is distinct from cytosine and is clearly not uracil. While it may be an artifact of acid-high temperature hydrolysis, its consistent appearance even at different concentrations of acid suggests that it may be a base constituent pre-existing in the nucleic acid. In this connection it might be pointed out that 5-methylcytosine was reported by Johnson and Coghill as a constituent of the deoxyribonucleic acid of the tubercle bacillus (8). Furthermore, it is true that epicytosine stands in the same relation to cytosine with respect both to its absorption spectrum and its mobility in butanol that thymine (5-methyluracil) does to uracil. More than this cannot be said until further study of epicytosine has been made.

Another abnormality that has occasionally been noted is the appearance of an "epiguanine," traveling about 0.7 as fast as cytosine and having a double band spectrum somewhat like guanine. This substance is believed to be a derivative of guanine, probably an artifact, formed in the hydrolysis of certain deoxyribonucleic acid preparations.

Routine Identification of Major Constituents—For less rigorous identification of the isolated fractions, use is made of certain characteristic features of the absorption at a few chosen points in the absorption spectra. The distinguishing features which have been used to differentiate the bases are indicated in Table III. Mention should again be made that guanine absorption responds to small fluctuations in pH; so that quantitative data for this base are not so readily reproducible.

Determination of Composition of Binary Mixtures Obtained between Absorption Peaks—In some places between the peaks, optical density of the eluate at 260 $m\mu$ may be as low as 0.10 or less, and identification of such small amounts of bases by the methods outlined heretofore would be impossible. Furthermore, if the solvent migration has not been carried far enough, or if the paper segments have been cut too wide, these regions may carry small amounts of two individual bases derived from the absorption peaks on either side of the spot in question. In case information about these weaker solutions is desired, a quantitative analysis can be made, based upon the assumption that they contain no ultraviolet-absorbing substances other than one or both of these two bases.

Simultaneous equations can be derived for any two bases at any two wave-lengths from the data in Table II. Since, however, the accuracy of such a calculation depends upon having a maximum of difference between the absorption contributions of the two components, certain wave-lengths are far more useful than others. Briefly, use is made of the following facts: thymine or uracil absorption is shifted much farther into the longer wave-

lengths than that of adenine by addition of alkali, and absorption by cytosine extends farther than that of uracil into the longer wave-lengths in neutral, and especially in acid, solution.

In the calculations that follow, total absolute absorption (corrected for paper and cuvette blanks) is indicated by the symbols used in Table III. Partial absorption calculated for a given component is further identified by

TABLE III
Characteristic Absorption Values of Individual Purines and Pyrimidines

	$\frac{N_{275}}{N_{265}}$	$\frac{N_{285}}{N_{265}}$	$\frac{OH_{295}}{OH_{265}}$	Other characteristics
Thymine	0.74	0.56	1.03	$N_{280}/N_{260} = 0.53$; $OH_{max.}$ at 290 $m\mu$
Adenine	0.34	0.36	0.05	$N_{280}/N_{260} = 0.12$; OH shift small, H shift > in uracil or thymine
Uracil	0.46	0.32	0.89	$N_{280}/N_{260} = 0.17$; $OH_{max.}$ at 285 $m\mu$
Cytosine	0.82	1.46	0.68	$H_{max.} \gg N_{min.}$; $N_{min.} \gg H_{min.}$ or $OH_{min.}$; H shifts toward long wave
Guanine	1.1	0.52	0.50	2 bands, N_{245} and N_{275} ; sensitive to pH
Thymidine (desoxyriboside)	0.85	0.67	0.24	Like thymine except OH shift less; $OH_{min.} \gg N_{min.}$
Adenosine	0.42	0.32	0.02	Like adenine but not affected by alkali
Cytidine	1.12	1.25	0.37	Like cytosine except OH shift toward short wave; $OH_{min.} > N_{min.} > H_{min.}$
Guanosine	0.88	0.35	0.16	Like guanine except OH shift smaller
Guanine desoxyriboside	0.89	0.34	0.16	Like guanosine
Xanthine	0.87	0.93	0.91	
Hypoxanthine	0.50	0.42	0.26	
2-Amino uracil (isocytosine)	1.07	0.26	0.32	

OH, N, and H refer to absolute absorption in alkaline, neutral, and acid solutions, respectively; the subscript indicates the wave-length in $m\mu$ of the maximum or minimum.

a subscript initial of the substance concerned. Thus, OH_{265T} indicates that portion of the absorption in alkaline solution at 265 $m\mu$ which is due to thymine.

(a) *Thymine-Adenine Mixtures*—For this pair the absorption is determined in alkaline solution at 290, 280, and 265 $m\mu$ (absorption at 260 $m\mu$ in neutral solution being already known).

Since $OH_{265T} = 0.79 OH_{290T}$, and since OH_{290A} is negligible, $OH_{265T} = 0.79$

OH_{280} , and $\text{OH}_{265\text{A}} = \text{OH}_{265} - \text{OH}_{265\text{T}}$ (by assumption). As a check upon these calculated values, compute $\text{OH}_{280} = 1.21 \text{ OH}_{265\text{T}} + 0.48 \text{ OH}_{265\text{A}}$, comparing this computed value with the observed total (corrected) absorption at $280 \text{ m}\mu$. Since the aim is to determine the partial absorptions at N_{260} , the following factors¹ are used: $\text{N}_{260\text{T}} = 1.78 \text{ OH}_{265\text{T}}$ and $\text{N}_{260\text{A}} = 1.12 \text{ OH}_{265\text{A}}$. The sum of these should very nearly equal the observed (corrected) N_{260} ; but even if it deviates from the latter somewhat, the ratio probably defines quite accurately the proportion of the two substances. If only one substance is present, the value for the other comes out as a very small positive or negative one. A large negative value indicates impurities not allowed for in the underlying assumption.

(b) *Adenine-Uracil Mixtures*—The procedure is the same as for the mixture just discussed, except that the numerical coefficients are different. Readings are at 290, 280, and $265 \text{ m}\mu$ in alkali. We find $\text{OH}_{265\text{U}} = 0.87 \text{ OH}_{290}$; $\text{OH}_{265\text{A}} = \text{OH}_{265} - \text{OH}_{265\text{U}}$. Checked by comparison at $280 \text{ m}\mu$, $\text{OH}_{280} = 1.29 \text{ OH}_{265\text{U}} + 0.48 \text{ OH}_{265\text{A}}$, and calculated back to neutral solution, $\text{N}_{260\text{U}} = 1.74 \text{ OH}_{265\text{U}}$ and as before, $\text{N}_{260\text{A}} = 1.12 \text{ OH}_{265\text{A}}$.

(c) *Uracil-Cytosine Mixtures*—Absorption is determined at 245, 260, and $280 \text{ m}\mu$ in neutral solution. $\text{N}_{260\text{C}} = 2.38 \text{ N}_{280} - 0.42 \text{ N}_{260}$, $\text{N}_{260\text{U}}$ being calculated by difference. To check, calculate $\text{N}_{245} = 0.775 \text{ N}_{260\text{C}} + 0.655 \text{ N}_{260\text{U}}$. More accurate results are obtained in acid solution, observations being made at 290, 265, and $245 \text{ m}\mu$: $\text{H}_{265\text{C}} = 1.73 \text{ H}_{290} - 0.019 \text{ H}_{265}$; $\text{H}_{265\text{U}} = \text{H}_{265} - \text{H}_{265\text{C}}$. Checked by $\text{H}_{245} = 0.235 \text{ H}_{265\text{C}} + 0.735 \text{ H}_{265\text{U}}$; and calculated back by $\text{N}_{260\text{C}} = 0.725 \text{ H}_{265\text{C}}$ and $\text{N}_{260\text{U}} = 1.11 \text{ H}_{265\text{U}}$.

(d) *Guanine-Nucleotide Mixtures*—Of the substances remaining in the initial spot, guanine is the principal one whose absorption is shifted by alkali. The increase in absorption at $275 \text{ m}\mu$ occasioned by adding alkali is very roughly 15 per cent of $\text{N}_{260\text{G}}$.

(e) *Other Mixtures*—Thymine and uracil are not near together on the paper and indeed appear not to be found closely associated in nature, but if necessary they may be distinguished at $310 \text{ m}\mu$ in alkaline solution.

Adenosine, adenine riboside, may occur on the paper near uracil. It may be distinguished from uracil exactly as is adenine itself, and from adenine by its total lack of absorption shift in alkali around 265 to $285 \text{ m}\mu$.

Thymidine, thymine desoxyriboside, travels in *n*-butyl alcohol very close to thymine. The nucleoside absorption is almost unaffected by alkali and may be estimated from the fact that OH_{300} of a mixture is almost entirely due to the free thymine.

Guanosine and cytidine have absorption spectra very similar to their

¹ In this and other similar calculations, the coefficients include a small empirical correction in which the 2 per cent increase of volume occasioned when 3 cc. of neutral solution were made alkaline or acid as described is automatically taken into account.

parent bases, but appear widely separated from them in the paper. Uridine has not yet been studied.

(f) *Results*—Application of the equations indicated above to mixtures encountered in an actual paper strip experiment gave the results indicated in Table IV. It will be seen that the several mixtures could be analyzed in

TABLE IV
Quantitative Analysis of Dilute Binary Mixtures

Optical density		Thymine-adenine mixtures			Uracil-adenine mixtures		
		Mixture I	Mixture II	Mixture III	Mixture I	Mixture II	Mixture III
Data	OH ₂₆₅	0.006	0.008	0.058	0.053	0.019	0.000
	OH ₂₆₅	0.195	0.072	0.051	0.067	0.063	0.059
Results	OH _{265A}	0.190	0.066	0.005	0.021	0.046	0.059
	OH _{265T}	0.005	0.006	0.046			
	OH _{265U}				0.046	0.017	0.000
Check							
Calculated	OH ₂₆₅	0.097	0.039	0.058	0.069	0.044	0.028
Observed		0.099	0.042	0.057	0.072	0.046	0.028
Calculated	N ₂₆₅	0.222	0.085	0.088	0.104	0.082	0.066
Observed		0.222	0.092	0.086	0.105	0.081	0.063
Cytosine-uracil mixtures							
		Mixture I		Mixture II		Mixture III	
Data	H ₂₆₅	0.089		0.043		0.005	
	H ₂₆₅	0.163		0.172		0.100	
Results	H _{265C}	0.151		0.071		0.007	
	H _{265U}	0.012		0.101		0.093	
Check							
Calculated	H ₂₆₅	0.045		0.091		0.070	
Observed		0.052		0.085		0.076	
Calculated	N ₂₆₅	0.122		0.163		0.108	
Observed		0.114		0.159		0.108	

The figures represent corrected optical density values, respectively. OH, N, and H refer to alkaline, neutral, and acid solutions; OH₂₆₅ represents the total absorption at 265 mμ in alkaline solution; OH_{265A}, the partial absorption due to adenine at 265 mμ in alkaline solution; etc.

terms of two partial absorption spectra which allow successful prediction of the absorption value at another reference wave-length. The broken line curves of Figs. 1 and 2 are based upon similar analyses in other cases. It may be pointed out that the solutions described in Table IV contained 0.6 to 2.5 γ (average 1.5 γ) of total bases per cc.

Quantitative Recovery of Bases—Since only negligible quantities of any

base are present outside the region immediately surrounding its peak absorption, the recovered quantity can be immediately ascertained by totaling the absorption found in this region. For such purposes it is best to analyze, as just described, all solutions which may conceivably be mixtures. The sum of total absorptions of the pure solutions and of the partial absorptions in mixtures is the absorption that would have been obtained if all the recovered base were present in one portion of eluting solvent (in this work, 3.5 cc.). This is the sum of a series of definite integrals, which could also be determined, far less accurately however, from the area under the corresponding peak on the smoothed chromatogram plot.

Table V illustrates the recovery of bases from strips carrying single compounds and also known mixtures. The figures are given in micrograms

TABLE V
Quantitative Recovery of Purines and Pyrimidines

Preparation analyzed		Thymine	Adenine	Uracil	Cytosine	Guanine
		γ	γ	γ	γ	γ
Pure substances	Theory	90	89	94	91	
	Found	79	93	92	86	
Mixture I	Theory	84	52	66	84	
	Found	81	50	65	79	
Mixture II	Theory	72	71	75	72	
	Found	67	69	73	69	

2 mg. yeast nucleic acid hydrolyzed 2 hrs. at 120° with hydrochloric acid						
<i>normality of acid</i>						
0.4	0	123	7	9	125	
0.7	0	112	11	9	125	
2.4	0	112	54	18	140	
6	0	132	117	52	125	

of anhydrous bases.² There is clear evidence that the recovery is essentially quantitative, errors probably being caused in large part by some uncertainty and variability in the corrections due to substances present in the paper.

Study of Nucleic Acid Hydrolysis—As a practical test of the method, equal samples of yeast nucleic acid were hydrolyzed at 120° with varying concentrations of hydrochloric acid. The hydrolysates were concentrated to dryness *in vacuo* to remove hydrochloric acid, neutralized, and applied in known quantities to the paper. After migration with butyl alcohol, the bases were recovered to the extent shown in Table V. It will be noted that

² Since the theoretical, expected values are based upon absorption data, the agreement between theoretical and actual recoveries is independent of the specific extinction coefficients used.

free adenine is liberated rapidly at acid concentrations too low to liberate appreciable quantities of the uracil or cytosine. Appreciable quantities of nucleosides are found in such partial hydrolysates. At higher acid concentrations the nucleosides are decreased and uracil and, finally, cytosine appear in free form. The maximum amount of cytosine indicated in Table V may still represent incomplete recovery. Guanine is apparently liberated in free form as rapidly as the adenine; otherwise the nucleoside, which travels about 0.4 as fast as cytosine, would have been observed. These results are in keeping with the known greater lability of the purine ribosides compared with the pyrimidine ribosides.

DISCUSSION

The method outlined above appears to provide a means of estimating with some accuracy the constituent purine and pyrimidine bases, and probably the nucleosides, in hydrolysates of nucleic acids. The spectrophotometric standards given herewith make it possible to ascertain the identity and purity of recovered bases and the composition of binary mixtures that may be encountered. These aims have not been fulfilled to anywhere near the same extent in the paper chromatography of amino acids. Nevertheless, it should be pointed out that the present method, even when used for qualitative purposes, can be somewhat tedious, and the careful quantitative investigation of twenty or thirty strip segments for one single analysis may require several hours. Furthermore, it is not a simple matter to hydrolyze quantitatively a nucleic acid preparation without encountering some decomposition of the bases themselves. As already mentioned, two anomalous bases have been found in hydrolysates of certain desoxyribonucleic acid preparations, and one of them is believed to be a degradation product of guanine, or some other artifact. It is a fortunate circumstance that sugar constituents contribute very little to ultra-violet absorption either before or after hydrolysis.

In any case, the quantitative separation on paper strips should provide a means of judging whether the proportion of individual bases varies from one nucleic acid to another. In addition, if nucleic acids differ in the nature or arrangement of their internucleotide linkages, the rate at which individual bases are liberated during chemical or enzymatic hydrolysis may prove to be characteristic of individual modes of combination present. Investigation of residual fractions and fragments released during enzymatic hydrolysis (see (9)) should be rendered more convenient by the techniques given above.

Since this manuscript was completed, an abstract by Vischer and Chargaff (10) has appeared, indicating that these workers have now extended paper chromatography to the pyrimidines uracil and thymine.

They also now employ solvents not absorbing ultraviolet light and have applied the separation to quantitative analysis of nucleic acids. Their procedure is doubtless rather similar to the one reported here, although purines and pyrimidines are apparently determined in separate hydrolysates and in different solvents. These workers mention the conversion of cytosine to uracil during acid hydrolysis, which has so far not been observed in this laboratory, but their experience with hydrolysates of different nucleic acids appears to be more extensive at the present time.

The author wishes to acknowledge the patient and conscientious assistance of Margaret Brophy which greatly aided in the development of this method.

SUMMARY

1. By paper strip chromatography in a butyl alcohol system, cytosine, uracil, adenine, and thymine may be isolated from mixtures and from hydrolysates of nucleic acids. Guanine can also be recovered under favorable circumstances.

2. The nucleosides that have been examined, cytidine, guanosine, adenosine, and thymidine, can also be separated from each other and from most of the free bases in the same system.

3. The isolated substances can be identified and their quantity determined by ultraviolet spectrophotometry. The substances are normally recovered in highly purified form if the migration is carried far enough.

4. If binary mixtures are encountered, their composition may be quantitatively determined.

5. Chromatography of partial hydrolysates of nucleic acids reveals the rate of liberation of individual bases and nucleosides.

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THE EFFECT OF CHICK EMBRYO EXTRACT ON THE GROWTH AND MORPHOLOGY OF TUBERCLE BACILLI

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PLATE 16

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Minced embryonic tissue of chicken is known to facilitate growth of tubercle bacilli in synthetic media. Thus Friedmann obtained faster and more abundant growth of both human and bovine strains by adding chick embryo tissue to a basal medium (6). Soltys cultivated avian strains in Tyrode solution containing 2 per cent of chick embryo pulp. While the human and bovine strains studied in his experiments multiplied in the first subculture in this medium, they failed to grow in subsequent transplants (8).

The present publication describes the effect of embryo extracts on the rate of growth, morphology, and virulence of tubercle bacilli cultivated in the media recently developed in this laboratory.

EXPERIMENTAL

Methods.—The culture media used were those described by Dubos and Middlebrook (5). In addition to mineral salts, casein hydrolysate, and serum albumin, these media contain 0.005 per cent oleic acid (medium 1) or 0.05 per cent of the water-dispersible ester of oleic acid Tween 80 (medium 2). 11-day-old chick embryos were removed aseptically from the eggs, washed in distilled water, and minced in a Waring blender following addition of 1.5 cc. distilled water per embryo. The embryo pulp was centrifuged at 4°C. for 30 minutes at 3500 R.P.M. and the supernatant fluid was used,—chicken embryo extract (CEE). Extracts of muscle, lung, kidney, and spleen were prepared by a similar technique. The tissue extracts were added under aseptic conditions to the culture medium distributed in 5 cc. amounts in Pyrex glass tubes 25 mm. in diameter.

Most of the experiments were carried out with the human strains of tubercle bacilli H37Ra (avirulent) and H37Rv (virulent).¹ A few bovine and avian strains have also been tested and found to behave essentially like the human strains. The standard inoculum corresponded to a final 10^{-4} dilution of a fully grown culture in Tween-albumin medium (approximately 3×10^{-4} mg. dry weight bacilli per cc. of medium).

Effect of CEE on Growth Rate.—The data presented in Table I summarize the plan and results of a typical experiment. They show that addition of 0.5

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¹ These strains were originally obtained through the courtesy of Dr. W. Steenken of the Trudeau Sanatorium. They have been subcultured in our laboratory in the Tween-albumin medium.

to 1.0 per cent of CEE to the oleic acid-albumin medium renders growth more abundant and more rapidly detectable than in the control media not containing the extract. This was particularly striking when small inocula were

TABLE I

The Effect of Chick Embryo Extract on the Growth of Tubercle Bacilli in Oleic Acid-Albumin Medium

Final concentration of CEE	H37Ra*			H37Rv*		
	Time after inoculation, days			Time after inoculation, days		
	3	6	12	3	6	12
<i>Per cent</i>						
0	1†	4†	6†	2†	5†	7†
1.0	4	6	8	5	8	>8
0.5	4	6	7	5	7	8
0.25	2	5	7	3	6	7
0.125	1	5	6	2	6	7

* Inoculum: 5×10^{-4} mg. bacilli per cc. of medium.

† The amount of growth is recorded in terms of turbidity estimated visually according to an arbitrary scale from 0 (no growth) to 8 (growth corresponding to approximately 0.4 mg. dry weight of bacilli per cc. of medium).

TABLE II

Influence of Size of the Inoculum on the Growth-Promoting Effect of Chick Embryo Extract

Final concentration of CEE	H37Rv Inoculum (mg. dry bacilli per cc. medium)								
	3 × 10 ⁻⁴			3 × 10 ⁻⁵			3 × 10 ⁻⁶		
	Growth after varying incubation time, days								
	3	6	12	3	6	12	3	6	12
per cent									
0	2*	5*	7*	1*	2*	3*	0*	1*	1*
1.0	5	7	>8	4	6	7	1	3	3
0.5	5	7	8	2	5	6	0	2	3
0.25	2	6	7	1	3	4	0	2	2
0.125	2	5	6	1	3	3	0	1	1

* Symbols same as in Table I.

used (Table II). Although no quantitative study has been made of the rate of bacterial multiplication, it appears from microscopic observations and from the increase in turbidity of the culture that the most pronounced effect of CEE takes place during the logarithmic phase of growth and that the lag phase is not markedly shortened. The observation that the maximum yield of bacil-

lary growth is reached earlier in the presence of the extract is in agreement with findings reported by others (6, 8).

Effect of CEE on Bacterial Morphology.—Middlebrook *et al.* (7) observed a correlation between the virulence of different strains of mammalian tubercle bacilli and the pattern of growth of these strains—a pattern due to the formation of bacillary cords. The more virulent a strain, the more pronounced was its ability to grow in the form of bacillary cords (serpentine pattern of growth). It was noted during the present work that addition of CEE to oleic acid-albumin medium caused the bacilli to organize themselves in cords which were longer and more dense than those formed in the absence

TABLE III

Effect on Male Swiss Albino Mice of Intravenous Inoculation of Tubercle Bacilli, H37R_o (Virulent) and H37R_a (Avirulent), Grown in the Presence or Absence of Chick Embryo Extract
(0.03 mg. dry weight of bacilli was inoculated into each animal.)

Inoculum 0.03 mg. of	No. of mice	Death and survival											
		S+	S+	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-
H37R _a	12	S+	S+	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-
H37R _a (CEE)*	"	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S-
H37R _v	"	D43	D45	D47	D47	D50	D50	D51	D51	S+	S+	S+	S+
H37R _v (CEE)*	"	D21	D21	D21	D27	D39	D39	D40	D40	D43	D48	D48	S+

D, death; the number indicates the number of days after inoculation at which death occurred.

S, survival for a period of 57 days, at which time the animals were sacrificed.

The symbol + means that tuberculous lesions were present in the lungs.

The symbol - means that no pulmonary lesions could be detected macroscopically.

* These cultures were grown in the presence of chick embryo extract.

of the extract and within which the bacterial cells were arranged strictly in parallel (Figs. 3 and 4).

Whereas in the case of the virulent variant H37R_v the embryo extract caused only a quantitative difference in cord formation, differences of a qualitative order were observed with the avirulent variant. In ordinary media, the avirulent bacilli always grew in unoriented clumps, but they formed well defined cords in the presence of CEE (Figs. 1 and 2). However, the fact that the avirulent bacilli always remain less acid-fast than the virulent forms facilitates their identification even when they grow in the form of cords.

Experiment has shown that cord formation by H37R_a in the presence of CEE is not a transmissible modification but occurs only if enough extract to induce it is present in the medium during growth. Growth assumes again the usual unoriented pattern as soon as the strain is returned to ordinary culture media devoid of CEE.

The ability of CEE to enhance or induce cord formation is best tested in the oleic acid-albumin medium. A more striking effect is observed in Tween 80 media but it is due to artifacts. The lipase present in CEE brings about hydrolytic destruction of the surface-active ester Tween 80 (1-3) and prevents the latter from inhibiting cord formation. In the case of virulent strains growing in Tween-albumin medium therefore, CEE greatly enhances cord formation by destroying the dispersing effect of Tween, but this unspecific effect is fundamentally different from the one mentioned above which occurs in oleic acid-albumin medium.

The Effect of CEE on Virulence.—As earlier studies have established a correlation between cord formation and virulence, it was of interest to study the relative virulence of strains H37Ra and H37Rv grown in the absence and in the presence of CEE. The results of experimental infections of Swiss albino mice with these cultures are summarized in Table III.

The organisms used in this experiment were grown in a medium containing 0.02 per cent Triton A20 as a wetting agent instead of Tween 80 (4). Triton A20 is not hydrolyzed by any enzyme present in CEE and, unlike Tween 80, therefore, retains its dispersing effect in media containing the extract. In consequence, it is possible to obtain in Triton A20 media cultures which consist of cords of approximately the same length, irrespective of the presence of the embryonic extract. This fact may be of some importance in assuring uniformity in the establishment of the pulmonary lesions.

The results presented in Table III suggest that the virulence of H37Rv is slightly increased when the culture is grown in a medium containing CEE. The mice infected with these cultures died earlier than those injected with the same amount of H37Rv growing without the extract. In both groups, however, the animals surviving after 57 days were extremely emaciated and probably would have died within a short time as necropsy revealed extensive confluent pulmonary lesions.

As shown in Table III, some tuberculous lesions were found in the lungs of mice infected with the H37Ra strains. These lesions were small and circumscribed, and the animals appeared in excellent condition and gained weight.

Although the bacilli grown in the presence of CEE seem to have produced more lesions of this type, further study is required to evaluate the significance of these differences. Suffice it to mention here that in a further experiment mice infected with H37Ra and injected daily with CEE (0.2 cc. intraperitoneally) failed to exhibit tuberculous pulmonary lesions when sacrificed 4 weeks after infection.

Characteristics of the Active Components of CEE.—Aqueous extracts of chick embryo lose their ability to enhance and modify the growth of H37Ra slowly at ice box temperature and become inactive within 3 to 5 weeks. Activity

survives somewhat longer but not indefinitely at lower temperature. Heating to 100°C. reduces the activity about 50 per cent within 3 minutes and brings about complete inactivation within 6 minutes.

The active principle of CEE does not dialyze through cellophane membranes. It is not soluble in ether, ether-alcohol, alcohol, or acetone at neutral or acid pH. These properties appear compatible with those of a labile substance of a high molecular weight.

Extraction of the minced embryonic tissue with an excess of water for 1 hour at 4°C. removes all activity from it. The chick embryos used in the experiments reported above were 11 days old; younger and older embryos yielded less active material and extracts from 17-day-old embryos were nearly inactive. Extracts prepared from rabbit testicles, from mouse brain, liver, and spleen, from leucocytes of man and rabbits, guinea pigs, and mice, and from egg yolk, failed to show any effect on cord formation although they had some enhancing effect on the growth rate of tubercle bacilli.

DISCUSSION

Aqueous extracts of chick embryo exert a dual effect on tubercle bacilli: (a) they accelerate and increase growth; (b) they enhance the tendency of the bacilli to exhibit the serpentine pattern of growth. It is not known whether these effects are due to one or to several active principles.

Enhancement of growth could also be obtained with other tissue extracts devoid of any effect on cord formation, but in no case was it as pronounced as that obtained with the embryo extract. The fact that the ability of virulent organisms to grow in the form of cords is enhanced by CEE suggests the possibility that the extract supplies a factor similar to that synthesized by the virulent organisms. As the avirulent variants which normally do not form cords can do this in the presence of CEE, one may assume that these variants have lost the ability to synthesize specific metabolic products essential for cord formation but retain the potential property to grow in the form of cords when the essential metabolites are provided.

The ability of CEE to induce cord formation by the avirulent variant of human tubercle bacilli may explain the fact that H37Ra, which is avirulent for all experimental animals, can produce, on the chorioallantoic membrane of the chick embryo, lesions which differ only quantitatively from those caused by the virulent strain. In our experience, it has not been possible to cause a permanent reversion to the pattern of growth of the virulent form by prolonged serial cultivation of the H37Ra strain in media containing CEE.

SUMMARY

Aqueous extracts of 11-day-old chick embryos enhance the growth of tubercle bacilli in oleic acid-albumin media.

These extracts also increase the tendency of virulent strains to exhibit the serpentine pattern of growth and confer this property on avirulent variants which normally grow unoriented in clumps.

Growth in the presence of chick embryo extract slightly increases the virulence of the virulent strains but does not confer virulence on the avirulent variants.

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EXPLANATION OF PLATE 16

The photographs were made by Mr. Julian Carlisle.

FIG. 1. Ziehl-Neelsen-stained preparation of 8-day-old culture of avirulent tubercle bacilli (H37Ra), grown in oleic acid-albumin medium. The bacilli lie helter-skelter in clumps. $\times 1090$.

FIG. 2. Ziehl-Neelsen-stained preparation of avirulent tubercle bacilli (H37Ra), grown in oleic acid-albumin medium containing 0.5 per cent chick embryo extract. The bacilli are arranged in parallel and form cords. $\times 1090$.

FIG. 3. Ziehl-Neelsen-stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid-albumin medium. The bacilli form cords. $\times 1090$.

FIG. 4. Ziehl-Neelsen-stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid-albumin medium containing 0.5 per cent chick embryo extract. The cords are more dense than in Fig. 3 and the parallel arrangement of the bacilli is more pronounced. $\times 1090$.



(Bloch: Chick embryo extract and tubercle bacilli)

THE NEOPLASTIC POTENTIALITIES OF MOUSE EMBRYO TISSUES

IV. LUNG ADENOMAS IN BABY MICE AS RESULT OF PRENATAL EXPOSURE TO URETHANE*

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PLATES 24 TO 28

(Received for publication, August 5, 1948)

In previous papers from this laboratory (1) experiments have been described which were made to learn how soon in the life of the organism cells possess the ability to become tumor cells. The neoplastic potentialities of various mouse embryo tissues, procured in the latter half of gestation, were tested by transplanting them to adults, together with methylcholanthrene. Tumors arose swiftly and in great diversity, yet it was questionable whether the cells exposed to the carcinogen were still in the embryonic state when they underwent neoplastic change, since the interval before the growths first became perceptible somewhat exceeded the time until birth, had the embryos been left undisturbed. If it had been possible to utilize the cells of very young embryos perhaps tumors could have been obtained within this period, but they did not survive the requisite exposure to methylcholanthrene, even when this was circumspectly injected into the "beads" along the uterus. Obviously for a decisive test of the neoplastic potentialities of embryo cells these must be exposed *in utero* to a carcinogen acting so speedily that its neoplastic effects will be evident almost at once. Recent authors who have produced pathological changes in mouse embryos with the Roentgen rays (2) have made no mention of tumor formation, and the polycyclic hydrocarbons fail to pass the placenta in effective quantity. It was recalled however that the injection of the highly diffusible hypnotic, urethane, into adult mice of strains liable to pulmonary adenomas in old age causes these growths to appear earlier than usual and in much greater number (3). We had employed such a strain of animals when testing with methylcholanthrene the neoplastic potentialities of transplanted fragments of embryo lung, and had noted that multiple adenomas formed within 2 to 3 weeks (4). Consequently urethane was now injected repeatedly into pregnant females, and their offspring were searched for adenomas. These were sometimes visible in 3-day-old animals, and often had attained a considerable size within 10 days, none appearing in controls. The urethane could have acted *in utero* for only a few hours, and it produced no visible damage in the lungs of

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the embryos exposed to it, which might have served as the basis for neoplastic changes occurring secondarily. All went to show that these changes were primary and took place prior to birth.

Law (5) has subjected embryonic tissues to the action of dibenzanthracene, injecting an olive oil solution of it directly into the amniotic sac of mouse embryos at the 15th day of gestation. The animals were killed when 200 days old on the average, and nearly all had lung tumors, in most cases multiple. While some of the growths may have been malignant the context of Law's paper indicates that the great majority were adenomas. The only other tumor developing was a fibrosarcoma of the skull in one instance.

Larsen has lately reported the presence of induced pulmonary adenomas in the 6-months-old offspring of A strain mice receiving urethane while pregnant (6). The work now described was done without knowledge of his. He found that urethane gave rise to few tumors in the young unless injected into the mother animals during the last 24 hours before parturition, and he noted that these latter showed many more growths than their young when all were examined together. His revealing observations will be considered in detail further on.

Materials and Methods

Mice of the C strain were mainly employed, as in the previous tests with transplanted embryo tissue. A large colony of the animals was available. In corollary several experiments were carried out with A mice raised in our laboratory.

The number of adenomas induced by urethane in adult mice is directly proportionate to the amount of the substance given (7). Hence it was injected into most of the pregnant females on several successive days, to a maximum of six, and in the greatest tolerable quantity. The precise duration of pregnancy was determined by the vaginal plug method in many instances, but palpation was relied upon in the majority to gain an idea of the age of the embryos. The injections were frequently begun as soon as these had pulmonary tissue for the urethane to act upon, that is to say round about the 12th day of gestation,¹ since our aim was to induce neoplastic changes as early as possible. Most of the females injected at that time either died or aborted as the injections were repeated, and our data have been procured in the main from those nearer term. Adult mice are known to differ widely in their tolerance of urethane.

At each injection the pregnant animal received, irrespective of its weight, 0.3 cc. of a 10 per cent solution of urethane in the subcutaneous tissue of the back. This produced 1 to 3 hours of apparent unconsciousness. The solution was very hypertonic because through a technical lapse the urethane had been dissolved in 0.9 per cent salt solution. Water bottles were used to supplement the fluid obtained from the diet of bread, milk, and lettuce. Not a few of the young born after the usual 21 days of gestation were puny and far below the normal weight, and these got their hair late and grew but slowly during several weeks. Ultimately

¹ We have been unable to find any account of when the lungs begin to form in the mouse. They are recognizable in embryos 12 days old. The tracheal anlage can then be seen pushing out from the foregut and ballooning into lung buds with primitive bronchi. It would be difficult to identify the lungs previous to this stage.

many litters were obtained however which resembled most of the controls in weight and vigor.

Some of the injected females were killed on the 18th to 20th day of gestation, and others on the 20th or 21st day, or when delivery had begun, in order to procure lungs which had not been exposed to postnatal influences. If the fetuses failed to breathe after removal from the uterus respiration was stimulated by dropping them repeatedly from a height of a few centimeters; and after they had taken a few breaths the chest was opened widely by lateral cuts, a flap including the sternum was everted, and the whole creature was plunged into acid Zenker's solution, for fixation of the pulmonary tissue *in situ*. Day-old mice were chilled in an ice box until insensible, and their lungs were fixed by the same procedure. Three-day- and 10-day-old young were killed by pushing in the cranium, after which a cord, already placed around the neck, was quickly tightened to prevent any aspiration of blood. The lungs were then dissected out and fixed as usual. By these procedures well distended pulmonary tissue was obtained, in which tiny adenomas could readily be perceived with the microscope. Mice 15 days old or older were chloroformed and the lungs were allowed to collapse to some extent prior to fixation, in order to reduce the number of sections to be examined. When the litters were large some of their members were killed 1 or 3 days after birth, in order to render the conditions more favorable to development of adenomas in the others; for the occurrence of these growths in adult mice is largely dependent upon sufficient food (8). Three groups of young animals, 3 days, 10 days, and 60 to 70 days old respectively, were searched intensively for adenomas, together with the appropriate controls. Many of the mother animals were killed and examined at the same time as their last surviving young.

Not a few of the 60 to 70-day-old offspring of urethanized mice had adenomas visible in the gross on the pleural surface, whereas none of the controls had any. They could not be seen on the pleura of 10-day-old animals, even at a magnification of $\times 17$, and hence the lungs of these and of 3-day- and 1-day-old individuals were searched *in toto* in serial sections $7.5\ \mu$ thick, as were also those of fetuses near term. The pulmonary tissue of certain of the older animals was searched in the same way, notably when the incidence of tumors in mothers and young was to be compared. The searching was done, section by section, at a magnification of 120 diameters in the case of mice 10 to 70 days old, and at 180 diameters in that of younger creatures. This serial scrutiny proved crucial in the case of adenomas just forming, because they were often simulated in individual sections by cell groups cut through at the bend of a blood vessel or bronchiole, or were mimicked by pleural infoldings.

The choice of stains, methylene blue and eosin, proved fortunate, for they made the tiny adenomas of young animals stand out from their surroundings, ordinary lung parenchyma appearing purplish-pink save for the nuclei, whereas the adenoma cells were basophilic, sky blue to dark blue, according to the intensity of the staining.

Findings in the 60 to 70-Day-Old Offspring of Urethanized Mothers

Pure breeds of mice differ widely in their liability to spontaneous adenomas. These appear soonest and are most frequent in animals of the A strain, the C strain ranking next but far behind. Andervont found them in 20 to 30 per cent of C mice over a year old (9), as nodules visible in the gross on the pleural surface,—the "peripheral index"; but in our colony they almost never manifest themselves in this way in individuals less than 9 months old, much older ones often showing none. They are nearly always solitary. No one has tested the response of C animals to urethane, but to methylcholanthrene it is notably slow. The tumors were still absent from Shimkin's animals 13

weeks after an intravenous injection of this hydrocarbon, and there were only 4 to a mouse after 20 weeks, whereas in adults of the A strain they averaged 25 to an animal after 6 weeks, 31 after 13 weeks, and 47 after 20 weeks (10).

These findings gave no ground for the supposition that urethane would produce adenomas swiftly in the offspring of urethanized C mice; only their presence in implants of embryo tissue which had been acted upon by methylcholanthrene made early examination of the young seem worth while. In serial sections of the lungs of a 30-day-old animal of the first litter from a urethanized mother a typical adenoma was encountered, and in the other mouse of this litter, killed 60 days after birth, there was a growth on the pleural surface visible to the unaided eye. Consequently we now set aside many newborn mice and examined them in the gross 60 to 70 days later. The controls were in general killed when slightly older, mostly after 70 to 100 days, which tipped the scales somewhat in their favor because the longer a mouse lives the greater is the likelihood that adenomas will appear spontaneously. In a total of 97 controls examined when 60 to 104 days old, no tumor was to be seen on the pleural surface, whereas adenomas were present in 21 of 80 individuals 60 to 70 days old from urethanized mothers,—just such tumors both in the gross and microscopically, as develop spontaneously in adults. They averaged a millimeter in diameter, but a few were as much as 2 mm. across, and in occasional individuals two or three of them were present. The lungs were remarkably free from equivocal nodules.

In one young animal out of a group of seven killed when 30 days old, from four litters born to urethanized females, an adenoma had developed that was visible in the gross. Its character was confirmed with the microscope.

The Growths in Mice 10 Days and 3 Days Old

Adenomas proved fairly frequent on microscopic examination of the lungs of 15-day-old animals from urethanized mothers, and hence an intensive search was made for them in serial sections from still younger individuals. Tables 1 and 2 give the findings in groups 10 days and 3 days old respectively. It will be seen that 10 out of 16 of the 10-day-old sucklings had adenomas, in some cases two or three. In none of the lungs from 16 control animals 10 days old was any structure found that could be classed as an adenoma with certainty, though the organs were searched in serial sections with equal care.

Only characteristic adenomas (Figs. 1-3) have been given place in Table 1, of 10-day sucklings. Some were surprisingly large, as much as 0.2 mm. across, and not infrequently compression of the adjacent parenchyma attested to their rapid growth² (Fig. 1). They had a

² Some authors have remarked upon the fact that the induced or spontaneous adenomas of adult mice seem to be mostly situated at the pleural surface; but of the 15 adenomas found in animals 10 days old nine were well down in the parenchyma. It was noteworthy in this relation that the pulmonary tissue next the biggest tumors frequently showed compression on the side toward the pleura (Fig. 1), and occasionally there was an outward bulge over

TABLE 1
Adenomas in 10-Day-Old Mice from Urethanized Mothers
(Only indubitable adenomas are included)

Urethane injections	Interval to birth	Mother No.	Adenomas	Time from first dose	Weight at death	Remarks
<i>No.</i>	<i>days</i>			<i>days</i>	<i>gm.</i>	
4	1	I	0	15	5	
		II	0	"	5	
		III	0	"	8	
6	1	IV	A-A A A 0	17	5.3 5 5 4.7	See Table 2 for litter mates
		V	A-A-A	"	4	" "
		VI	A A-A	"	7 6.6	" "
	2	VII	A 0	18	5.5 "	
	3	VIII	A 0 A-A A	19	5.5 " 5 4.5	
		C I	0 0		5.2 3.9	See Table 2 for litter mates
		C II	0		6.8	
		C III	0 0		6 5.5	
		C IV	0		6	
		C V	0		5.6	
		C VI	0 0		6.5 6	
		C VII	0 0 0		8.3 8.2 "	
		C VIII	0 0		4.5 5	
		C IX	0 0		4.5 4.7	
Controls						

Each A means an adenoma.

close general resemblance to the adenomas arising spontaneously in adult C animals and those induced in A mice with methylcholanthrene or urethane, and like them were either wholly glandular (Fig. 3) or partially compact (3, 11) (Fig. 2), their older central portion then being adenomatous. At some marginal spots the neoplastic cells formed a single layer on the walls of alveoli, as if extending along them with local heapings up.

Although the growths could easily be distinguished for what they were,—a recognition validated by study of the specimens from mice killed in later weeks,—they differed significantly in detail from the adenomas of adults. This matter will be considered further on.

To recognize the tumors in 3-day-old animals proved more difficult. Only three out of 10 of them had indubitable adenomas (Table 2), a single growth in each instance, but three others had what seemed to be early stages, wholly resembling those reported by Nettleship, Henshaw, and Meyer as appearing in urethanized adult mice (3), and comprehensively described by Grady and Stewart in their study of how adenomas come about after the intravenous injection of a colloidal suspension of methylcholanthrene (11).

The tumors were much less well developed than in the 10-day animals, as would follow from the brevity of the interval since the first urethane injection. The largest was found in the individual surviving for the longest time after the first injection of the mother (14 days), the next largest after the next longest time (11 days), while in the case of the least advanced growth only 10 days had elapsed. The largest (Figs. 4 and 5) lay deep in the lung, was markedly basophilic, and stood out sharply from the surrounding parenchyma. It consisted of the characteristic cuboidal cells, enclosing and partially filling a number of small spaces. A venule lay amidst it, as frequently happens in the forming adenomas of adults. It appeared multicentric where cut across, and its aspect suggested an origin from bronchiolar epithelium, but serial sections proved it to be a single growth lying well away from any bronchus or bronchiole. The lining of these latter stained a much paler blue.

Fig 6 shows the subpleural situation and discrete character of the growth found 11 days after the first exposure of the mother to urethane, and Fig. 7 reveals its characteristic morphology. Its cells are ranged in the usual single layer about small "acinar" spaces, and already it protrudes from the pleural surface. The parenchyma round about looks normal; as yet the cells lining the alveoli have undergone little flattening.

The 3-day-old animal which showed a growth only 10 days after the first giving of urethane had six litter mates and four of these were let live until 10 days after their birth, that is to say until 17 days after the first injection. Two had then a well developed adenoma each (Figs. 1 and 3), and a third had two of them (Table 1, offspring of mother IV). The growth in the 3-day-old animal consisted of a clump of cells, for the most part compact but covering part of an adjacent alveolar wall with a single layer (Fig. 10). They were sharply set off from their surroundings by their strong blue color and large, round, vesicular nuclei (Fig. 9). Mitoses were present. The clump had no direct connection with the epithelium of any bronchiole but lay well down in the parenchyma, and there was no cellular reaction round about it.

growths situated at some distance beneath it. The inference seems justified that a considerable proportion of the adenomas visible on the pleural surface of adult mice reach the surface secondarily as result of local pressure conditions as well as of enlargement. There is no need to invoke local differences in their initial distribution in the lung tissue.

The lungs of three other 3-day-old mice contained small discrete clumps of markedly basophilic cells with round, vesicular nuclei. They were solitary, and were situated in or on the wall of an alveolus. The instance pictured (Fig. 8) came from a sixth animal of the litter

TABLE 2
Adenomas in 3-Day-Old Mice from Urethanized Mothers

Urethane injections	Interval to birth	Mother No.	Adenomas	Time from first dose	Weight at death	Remarks
No.	days			days	gm.	
4	7	IX	A	14	2	
6	1	IV	0 A? A	10	2 1.8 1.9	See Table 1 for litter mates
		V	A?	"	2	" "
		VI	0 0 A?	"	2 1.9 1.9	" "
	2	X	A 0	11	1.9 1.9	
Controls		C I	0 0 0 0		1.7 1.6 1.6 1.5	See Table 1 for litter mates
		C X	0 0 A? 0		2 2 1.9 1.8	
		C XI	0 0		2.6 2.7	
		C XII	0		1.8	
		C XIII	A? 0		2 2.2	

A = characteristic adenoma. A? = dubious early stage.

of seven (Tables 1 and 2) dealt with in the last paragraph, the seventh proving negative. The clumps are queried in Table 2.

The Findings in Newborn Mice and in Embryos

From the relative size of the adenomas in 10-day- and 3-day-old individuals, it seemed likely that any encountered in still younger mice would consist of but

few cells. And in fact no indubitable adenomas have been come upon in 10 animals (from three litters) which were killed within 24 hours after birth, or in 14 embryos (from four litters) 18 to 20 days along. All the mothers had received six injections of urethane,—the last of them 1 to 3 days before parturition in the case of the young that came to term.

The lungs of mice less than 24 hours old have an alveolar lining that is but little flattened as yet, and often they are still partly atelectatic, a state of affairs rendering the recognition of adenomas almost impossible, as Tyzzer noted in his classical study of these growths in adults (12). The cells lining the alveoli of fetuses near term are basophilic and cuboidal, with round, vesicular nuclei, in other words closely resemble the cells of adenomas. It would manifestly be impossible to discern minute growths of this sort if situated in the parenchyma, but if they lay next the pleura or bulged it outward they might be perceived; and we have found what appears to be a forming growth (Figs. 11 and 12) in one of four fetuses removed from a female on the day after the last of six injections of urethane.

The mother animal had been selected by palpation as in mid-pregnancy, and hence the age of her fetuses was not precisely known; but the development of their lungs indicated that they were close to term, though they were only 22 mm. long as compared with 24 to 28 mm. for newborn mice from normal mothers. Mention has already been made of the frequent stunting effect of urethane on the embryos. Figs. 11 and 12 show that the problematic growth was discrete, bulging the pleura outwards, and that it consisted of cells with vesicular nuclei, ranged about an acinar (?) space. Nothing like it was found in the lungs of the other embryos, including three from the same litter, nor in those of any of the newborn mice.

The Findings in Sucklings of the A Strain

As already stated, adult mice of the A strain are far more liable to spontaneous adenomas than are those of the C breed (9), while furthermore the growths can be induced much more readily with methylcholanthrene (10). Hence we supposed that the offspring of urethanized A females might develop them earlier and in relatively great number. But on test an obstacle was met: the A animals injected during the latter half of pregnancy did very badly although much bigger than Cs at this time, and though only the standard amount of urethane (0.3 cc.) was given. Those receiving six injections either died or aborted or ate their young at birth, and so too with most of the mice which had four or five injections, the few that survived being so close to term at the time of the last as to give birth to their young on the day afterwards,—as did also the only animal that received three injections. It follows that the total interval between the first exposure of the young *in utero* to urethane and examination of them 3 days and 10 days after birth was, 7 to 9 days and 14 to 16 days respectively, that is to say was shorter than in the case of C mice. Furthermore the embryos had been exposed to much less urethane, both because of the fewer injections and because of the large bulk of the mother. Whether for these

reasons or no, the growths have been infrequent and tiny in individuals 10 days old (Figs. 13-15), actually comparable with those in 3-day-old C mice. For this reason no extensive study has been made of them. None was found in several A animals examined when 3 days old.

The Early Stages of Adenoma Formation

As already remarked, the early stages of the growths resembled those observed in adult mice given urethane or methylcholanthrene (3, 11).

First seen in the parenchyma were occasional discrete clumps of cuboidal or rounded cells situated in or on the walls of alveoli (Fig. 8), and with cytoplasm that stained an intense blue, sometimes very dark, contrasting so sharply with the hue of the normal parenchyma as to attract attention at low magnification. These cells had round or slightly oval vesicular nuclei, unlike those of ordinary alveolar elements, which are mostly dense, almost pyknotic, and more or less oblong. The clumps seemed punctate in origin and almost devoid of capillaries, unlike the richly vascularized alveolar tissue about them; yet their components looked very active. The growths of Figs. 9, 10, and 15 represent the earliest stage at which an adenoma gave definite signs of what it was. Here the cells have heaped up into a compact mass, but in Fig. 10 an adjacent alveolus is partly lined with them; mitotic figures can be seen. The tiny growth of Figs. 6 and 7 looks as if it had been adenomatous from the first. The tumor of Figs. 4 and 5 is expressive of further development. None of the growths was multicentric.

In late fetuses and sucklings up to 10 days old, of both the C and A breeds, spherical or discoid aggregates of cells were frequently found just beneath the pleura, which were at first taken for compact adenomas, and with the more reason because of the frequency with which the growths are found in this situation. They occurred irrespective of whether the mother had received urethane, and consisted of more or less cuboidal elements with round vesicular nuclei and cytoplasm staining blue,—cells not to be distinguished individually from those of adenomas. Sometimes the aggregates were spherical, lying tangential to the pleura amidst the lung substance, but more often they were discoid and protruded on its surface. Mitotic figures were frequent in them, far more so than in the lung parenchyma round about; and often the cells partially filled adjoining alveolar spaces. But it was noted that some of the masses, though appearing spherical in cross-section, were really fusiform, running through many sections, and furthermore that a thin membrane formed by reduplication of the pleura was attached to the surface either over them or near by; and the fact became evident that the masses were mere “mooring clumps” for membranes joining one lobe of the lung loosely to another. Fig. 16 shows a characteristic example. Frequently the clumps were situated at the sharp edge of a lobe or next it, that is to say at sites where adenomas are prone to occur; but now and then one was on the shoulder of a lobe or its rounded convexity. Occasionally a small lenticular mass of blue-staining cells like those composing a mooring clump was to be seen closed in between the layers of the membrane, well away from the lung surface. Even where the cells of a clump filled alveolar spaces, they were never ranged in the glandular pattern of mature adenomas; yet the fact remains that if no membrane had been attached next them they could not have been distinguished with certainty from compact tumors of such kind. For this reason we have accepted no subpleural growth as an adenoma unless it had typical features, and hence undoubtedly some have been omitted from Tables 1 and 2. The pleural nodule of Figs. 11 and 12 lay on the convexity of a lobe, had no membrane inserted over it, and an acinus seemed to have formed within it; yet because of the existence of “mooring clumps” its status is problematic. These are not infrequent in embryos.

Solitary, almost spherical giant cells 30 to 40 μ across, containing two or three vesicular nuclei, were present now and again on the alveolar walls of 3-day and 10-day animals, never more than two or three of them to a lung. None was found where adenomas were forming, and they seemed to have no relation to this process. Quite often in both C and A sucklings,—from control as well as urethanized mothers,—there were what may be called “polymorphonuclear balls,”—small, sharply demarcated, spherical aggregates of cells staining blue like adenoma cells, but with nuclei of horseshoe shape or resembling those of the blood polymorphonuclears, though coarser and less pyknotic. The cells had no granules, showed no division figures, and the balls formed of them appeared in excellent state, without sign of central necrosis. Usually they lay just outside some capillary in the alveolar wall, which appeared patent and normal. Wholly different in aspect from adenomas, their significance is not clear and their ultimate fate has not been traced. None has been come upon in embryos.

The Findings in Control Animals

It is singular that the literature contains no observations on how soon spontaneous adenomas begin to form. The scarcity of mitoses when they first come to attention in adult mice makes plain that their recent growth has been exceedingly slow, and it may well be that they originate early in life. Hence our diligent search for them in the control young of the present work,—the more careful because the tumors would doubtless be small, and perhaps solitary.

The lungs of one of 13 normal C mice 3 days old, examined *in toto* section by section, had a discrete clump of dark blue cells, in the parenchyma far from any bronchiole (Fig. 22); but both the cells and their nuclei were smaller and denser than those of the adenomas found at this time in the offspring of urethanized females. In another control mouse 3 days old several adjacent alveoli were almost filled with clumps of blue cells. Their nuclei were smaller than ordinary and oblong in some instances, yet the resemblance to a forming adenoma was great and at one spot the cells were ranked in two parallel rows (Fig. 23), as often happens in such growths. In the lungs of three out of 16 normal animals 10 days old small compact clumps have been found of basophilic cells wholly resembling the one pictured in Fig. 8 as coming from a 3-day-old mouse of an urethanized mother. There was only one such clump in each animal.

In sum, the findings give some support to the possibility that adenoma cells may have been present in the pulmonary tissue of the young normal mice. Their existence could not have been excluded in any case, since it would be impossible to identify them if scattered singly. This much is certain however, that the lungs of the controls contained no adenomas identifiable as such.

Origin of the Adenomas

Some workers are convinced that the adenomas called forth by urethane in adult mice arise on the basis of chronic inflammation of the pulmonary tissue, due either to the substance itself or to intercurrent infection. No sign of any such course of events has been observed in the young animals of the present work. Orr believes that the preliminary inflammation may have disappeared by the time the adenoma is well formed (13).

Urethane is known to produce ascites and to cause injury to several organs, largely in consequence of capillary damage. Orr reported that it set up chronic pulmonary inflammation in the outbred stock mice employed in his experiments, and Winchester and Higgins (14) found that it induced more or less pulmonary edema in animals of the C strain. The quantity of urethane injected into the pregnant females of the present work frequently exceeded the tolerable maximum, many of them dying; and often the fetuses and young of the surviving animals were abnormally small, as already mentioned, while occasionally the development of the lungs seemed retarded; yet nowhere were any local cellular anomalies other than adenomatous change perceptible in the pulmonary tissue. Furthermore there was no cellular reaction about the forming growths, as the figures sufficiently demonstrate.

The lungs providing material for the conclusion that adenomas are secondary to inflammation have generally been the subject of consolidating infections. The C strain is no exception in this latter respect. Mature animals of our colony often develop a consolidation which slowly involves most of the pulmonary tissue and eventually proves fatal. The disease is obviously infectious, spreading rapidly amongst cage-mates; but its cause is still undetermined. The lesions it produces have no resemblance to adenomas. The bronchioles seem to be first affected, cellular exudate accumulating within some of them; more and more are implicated; large and small mononuclear cells accumulate round about them and about the blood vessels; and the alveoli become filled with swollen, desquamated elements. The gross result is consolidation. In all these respects the findings are like those in the "grey virus disease" described by Andrewes (15). Some of the urethanized mother animals, killed with their young 60 or 70 days after parturition, had developed the malady and hence were discarded together with their offspring, but the lungs of the great majority appeared normal, save for adenomas, and those of the embryos examined and of the 1-day- and 3-day-old sucklings gave no microscopic evidence of the disease. In a single 10-day-old animal its beginnings were found.

Manifestly the best method to determine whether the consolidating disease prepared the way for the adenomas was to try to induce the latter in the young of a breed with healthy lungs. This was one reason for the tests of A mice. Our colony of them is remarkably free from pulmonary inflammations and the "lung disease" in special has never been found. Nevertheless adenomas arose in the young of females urethanized while pregnant. There was no cellular reaction about the growths (Figs. 13-15).

These facts, considered with what has already been said, prove that the adenomas induced in young mice were not secondary to pulmonary inflammation but primary in origin. Nettleship, Henshaw, and Larsen decided that the growths they induced with urethane in adult animals ordinarily arose from tissue which had undergone "little or no injury," and Grady and Stewart found no trace of preliminary pulmonary disorder in connection with the early stages of the adenomas due to methylcholanthrene.

Derivation of the Growths

Opinions have differed widely on whether pulmonary adenomas originate from alveolar cells or from the epithelium of the bronchial tree. None of the growths in sucklings had any direct connection with this latter; they were everywhere surrounded by parenchyma. All were arising, or appeared to have arisen, either from the alveolar wall or from elements of alveolar character lying

next the pleura, and the resemblance to bronchial epithelium was but slight. In the mother animals on the other hand, examined 70 days after parturition but of indeterminate age, and doubtless having in some instances "spontaneous" adenomas antedating the urethane injections, growths were not infrequently encountered which were composed of elements like those lining the small bronchioles (Fig. 17) and staining nearly as pink. The morphological resemblance was complete, except that the tumor cells were seldom markedly cylindrical, a difference which might have been consequent on pressure factors. In both instances a large proportion of the nuclei were round or slightly oval, and vesicular, as in the growths of sucklings, but many others were oval or oblong and almost pyknotic, often notably big and sometimes then with much more cytoplasm than usual about them. When oblong they were frequently ranked with their long axes parallel. All gradations between the two types were present. The resemblance to bronchiolar epithelium can be seen in Fig. 18. The growth providing this figure, made for ease of comparison, was exceptional in protruding into the lumen of the adjacent bronchiole; but other adenomas with identical features (Fig. 19) were wholly isolated amidst the lung parenchyma, as serial sections proved.

Amount of Exposure to Urethane as Determining the Incidence of Tumors

In Table 3 are listed all those cases in which the urethanized females were examined, as well as their remaining offspring, 60 to 70 days after parturition. The age of the females was not known, but they were sturdy multipara, several months old at the least, and some must have been well on the way toward having "spontaneous" adenomas, as has just been remarked. They, not their embryos, bore the immediate brunt of the urethane. On both these grounds one might have supposed that they would have had adenomas more often than their young, when all were eventually killed. And this was so, 10 of 14 mothers showing them in the gross on the pleural surface as compared with 10 of 49 young. Yet they were few at most in the mothers and usually solitary. Repetition of the injections had but a slight effect; in only two of five mother animals which had received four or six injections were there definitely more tumors than in those which had received but one.

Larsen noted that the number of adenomas present after 6 months in the offspring of A mice receiving urethane while pregnant largely depended upon when the substance had been given (6). There were at least five times as many when it had been injected during the final 24 hours of gestation as when it had been administered earlier, and furthermore every animal had them, instead of a large proportion. The reason, Larsen concluded, was that more blood carrying urethane reached the fetal lungs in the final hours before birth. No such differences with time of injection have been perceptible in the present experiments. The microscopic findings in 10-day-old animals carry some weight in

TABLE 3
Peripheral Adenomas in Mothers Urethanized While Pregnant and in Their Offspring
 (Findings 60 to 70 days after parturition)

Urethane injections	Interval to birth	Mother	Offspring		Adenomas		Remarks
			Age	Mouse	Presence	Size	
No.	days	days		mm.			
		A			0		
	2		70	a	+	1	
				b	0		
				c	+	1	
				d	+	1	
				e	+	1-1	
	1	B			0		
			70	a	+	2	
				b	+	1-1	
				c,d	0		
2	6	C			+	1	
			67	a,b,c,d,e,f	0		
		D			+	1	
			67	a	+	0.5	
				b,c,d,e	0		
	1	E			0		
			70	a	0		
		F			0		
			70	a,b,c,d,e,f	0		
3	1	G			+	1	
			70	a	+	1.5	
				b,c	0		
		H			+	1-1	
			70	a	0		
	1	I			+	0.5	
			70	a,b,c,d,e	0		
		J			+	0.3	
			60	a	+	0.3	
		K			+	1-1-1-1-1	
4	1		70	a,b,c,d	0		Carcinoma in other lung
		L			+	1	
			70	a	+	1	
				b	0		
		M			+	1-1	
	4		70	a	+	2	
				b,c,d,e	0		
		N			+	1-1-1-1-1-1	
			70	a	0		
6	2						
			70	a	0		

this relation because their entire lungs were examined microscopically. It will be seen (Table 1) that the incidence of adenomas in the litters from mothers getting six injections of urethane was only dubiously larger when the last had been given within 24 hours of parturition than when it had been given 2 or 3 days previously. The findings in the young killed 60 to 70 days after birth stress the same point. The offspring of females receiving four injections, the last within 24 hours of parturition, had no more tumors visible in the gross than those from mothers receiving the final injection 4 days previously (Table 3).

Nor did repetition of the injections into the mothers make adenomas any more frequent in the young. Actually the incidence of these growths in the offspring of the four mothers receiving urethane only once was considerably greater than it was in the young born to the eight animals getting it three to six times, seven out of 20 young mice in the first category having tumors visible in the gross as compared with four out of 22 in the second.

Mention has been made of the fact that a large proportion of the pregnant mice receiving urethane several times did badly and gave birth to young that were far under weight. Here was a possible reason for the differences just brought out. For Tannenbaum has shown that the appearance of spontaneous adenomas in adult animals can be checked or even prevented by underfeeding them (8); and the offspring of mothers getting urethane three to six times might have been more poorly nourished than those from females receiving it only once. But all the young of Table 3 appeared healthy when they were killed 60 to 70 days after birth, and while some were unusually small the two groups did not differ significantly. Of course early differences crucial to the development of adenomas might have been ironed out by this time, but the findings in the 10-day-old mice of Table 1 speak against the influence of any such differences. It will be seen from the weights there given that the individual with most growths (offspring of mother V) was the smallest of the seven killed 17 days after a single injection of urethane into the mother,—weighing 4 gm. as compared with a maximum of 7 gm. An animal having three peripheral adenomas, each a millimeter across when it was killed 72 days after birth from a female repeatedly injected with urethane weighed only 13 gm. at this time instead of the normal 20 to 24 gm., and it had weighed only 2.8 gm. when 10 days old and been almost devoid of hair then. It is not listed in Table 3 because its mother was not examined at the same time.

A better reason why repetition of the injections failed to result in more growths is perhaps to be had in the difficulty of inducing adenomas in adult animals of the C strain. No previous observations with urethane are on record, but methylcholanthrene gives rise to the growths very slowly in C mice, as already mentioned, and the number is small for a long time. Only toward the 20th week after intravenous injection of the hydrocarbon did they appear

in Shimkin's test, whereas in A mice there were more than 24 to an animal by the 6th week (10). They were never many in our C animals, and hence chance must have entered largely into the gross findings of Table 3. Yet the results cannot be wholly accounted for on the basis of these facts. The C embryos exposed to urethane in the mothers receiving but a single injection of it proved so responsive that adenomas followed in a large proportion of instances (Table 3), the growths arising very rapidly (Tables 1 and 2). Multiple injections failed to elicit them in any greater number. One injection into two pregnant females 2 days before parturition resulted in adenomas in six of their nine offspring, whereas three and four injections into five mothers, the last given within the final 24 hours of gestation, yielded growths in only two of 14 young (Table 3). These data, which go against all experience with adult mice, strongly suggest the existence of litter differences in the potentialities for adenoma formation. No sex differences were observed.

One might have supposed that the young of the two females receiving most urethane and themselves developing multiple adenomas in consequence (mothers K and N, Table 3) would have had growths most frequently. Actually their young had none,—at least none visible in the gross on the pleural surface. But the pleural index must sometimes be misleading when pulmonary tumors are few. Nevertheless Table 3 in its entirety seems to indicate that the offspring of individual C mothers developing adenomas in the gross after urethane injection possessed no greater tendency to such growths than the young of females in which none appeared. Using the pleural index Lynch was unable to obtain any evidence of a maternal influence on the incidence of pulmonary adenomas in tarred mice (16), and Bittner and Little found none in the case of the spontaneous growths (17).

Age as Affecting the Adenomas

Nearly everyone studying the adenomas of adult mice microscopically, whether spontaneous or induced, has remarked upon the scarcity of mitoses. Yet in the growths of the 10-day sucklings they were so abundant that often nearly every section showed them (Fig. 1), five being visible in a single high-power field of the microscope in one instance. There were other signs too of pronounced cellular activity. The adenomatous pattern was often ill-defined, and the individual cells were less differentiated and more basophilic than in the growths of adults (Figs. 1 and 2). The nuclei were almost uniform in size, round and vesicular, with little chromatin and this mostly margined; the cytoplasm was relatively scant.

The tumors of the mother animals killed 60 to 70 days after parturition were far more various. All were frankly adenomatous in arrangement and mitoses were few or wholly lacking (Table 4); many of the growths stained purple and now and then the color was pink. Occasionally one consisted

entirely of cells with vesicular nuclei like those of 10-day-old mice, but in most instances a considerable proportion of the nuclei stained a dark, even, almost pyknotic blue (Figs. 17-19), and often then they tended to be relatively large, and oval or oblong instead of round. Not infrequently when this was the case they had an unusual amount of cytoplasm about them (Fig. 17). Some of the cells with these features may have been intrusive, but the majority were ranged side by side with the ordinary elements having round, vesicular nuclei, and there were all gradations between the two. The differentiation of the neoplastic elements,—for such it obviously was,—culminated in cells which resembled those lining the bronchioles, though not quite so acidophilic (Fig. 17). Often they were in rows like these.

In sum, it was plain that the adenomas of 10-day-old mice consisted of actively multiplying cells, which for that reason, at least in part, were undergoing but little differentiation, whereas those of the mother animals were indolent and had largely differentiated.

A corroboratory indication of these contrasting states was found in the relative size of the growths encountered in mothers and young respectively after 60 to 70 days. Although the mothers were by then well along toward the age when some might have had spontaneous adenomas visible in the gross, and although they had received urethane directly, the largest of their tumors were only half as big as those in certain of the young (offspring of mothers B and M, Table 3). Indeed the average adenoma in these latter was as big as any in the mothers. Active proliferation must have kept on during some weeks for them to have reached such a size.

In order to learn whether cell multiplication was still continuing in any important degree comparative counts were made of the mitotic figures in some of the growths of mothers and young. The undertaking was not comprehensive; just enough was done to answer the question posed. The findings are given in Table 4.

It will be seen that no mitoses were present in the two adenomas, each a millimeter across, of mother M of Table 3, nor any in a smaller growth disclosed on serial section of the lungs. There were a few in the 2 mm. adenoma of one of the young from this mother, as also in another growth of considerable size which lay deep in its lung parenchyma. But evidently cell division had almost ceased, and this was the finding also in the notably big adenomas of other young animals, the progeny of mothers A and G. Some of the growths in these young showed no mitoses whatever, and their maximum frequency was no greater than in several of the tumors of mother K. It may be recalled that this animal was one of the two which had multiple adenomas visible in the gross, after the repeated injection of urethane, and that none of her four young showed any tumors (Table 3).

The conclusion seems warranted that proliferative activity in the adenomas

TABLE 4

Mitoses in the Adenomas of C Mice Urethanized While Pregnant and of Their Young
(Findings in growths present 60 to 70 days after parturition)

Days	Urethane injections	Mother	Young	Size of tumor	Cells examined	Mitoses		Remarks
						Actual No.	No. per thousand cells	
70	1	A		mm.	no.			Whole tumor searched
			a	1	3,000	0	0	
			e	2	24,000	0	0	
			"	1	42,000	2	0.05	
			"		40,000	1	0.03	
			"		18,500	1	0.05	Whole tumor searched
			"		10,400	0	0	Tumor partly lost
	3	G			9,400	1	0.1	Tumor partly lost
			a	1.5	44,500	0	0	
60	4	J	a	0.3				Growth in mother lost
			"	0.3	24,500	5	0.2	Whole tumor searched
70	4	K (Her four young were negative in the gross.)		1	33,400	4	0.12	Whole tumor searched
				1	20,500	2	0.1	Tumor partly lost
				1	19,200	3	0.16	Whole tumor searched
				1	18,800	0	0	" " "
				1	15,700	3	0.19	" " "
					14,000	4	0.29	" " "
					8,400	2	0.24	" " "
					4,350	3	0.7	" " "
		M		1	63,000	0	0	Whole tumor searched
				1	33,400	0	0	" " "
					12,100	0	0	
			a	2	598,400	19	0.03	Whole tumor searched
			"		61,200	2	0.03	" " "

The growths for which no size is recorded were either microscopic or had lain hidden in the parenchyma. All recognizable stages of mitosis were counted.

of the young animals had almost ceased by the time they were 60 to 70 days old, being by then nearly on a par with that in the mother animals. And there was substantial evidence to this effect in the morphology of the growths, which now exhibited not infrequently a differentiation as complete as that in

the tumors of adults. It was marked in some of the biggest adenomas of the young (Figs. 20 and 21). Growths in the same individual often differed widely in such respect, just as happens in adults.

DISCUSSION

The main aim of the present work, like that of other experiments previously reported (1), has been to learn whether the cells of embryos possess the ability to undergo neoplastic change. On first inspection the facts seem to leave no doubt of this for the lung, yet they cannot forthwith be accepted as proof.

First one must know whether the growths appearing in the offspring of urethanized mothers are true pulmonary adenomas, and if so, whether the latter are really tumors. On both points the answer is yes. The growths have been followed through every stage to the form found in adults, and they exhibit the familiar, distinctive characters of pulmonary adenomas. All workers with these latter are now agreed that they are genuine neoplasms. Both the spontaneous and the induced growths have been transplanted successfully (18), and while some have become carcinomatous on passage others have retained their initial traits. Adenomas are the first and most frequent tumors to arise from pieces of mouse embryo lung implanted with methylcholanthrene in the leg muscles of adults of the C strain (4), and several obtained in this way have been transplanted. One has now been propagated in five successive groups of adult hosts, and it has retained its original adenomatous morphology although in the course of months it forms huge tumors, killing the hosts. So convincing is the evidence that the adenomas are genuine neoplasms that we have deemed it unnecessary to try to transplant those obtained in sucklings. Doubtless for the same reason Larsen has reported no transplantations of the adenomas present in 6-months-old A mice from urethanized mothers (6).

So rapidly is urethane excreted that its direct effect can only have been exerted upon the embryos *in utero*, organisms that is to say which are protected from many extraneous influences, notably most of the infections. This state of affairs has provided opportunity to gain light on several moot points.

Much uncertainty has existed as to whether the adenomas arise from alveolar cells or from bronchial epithelium. Tyzzer, who first studied them comprehensively, concluded that they could be of either origin (12). Those we found in sucklings were wholly surrounded by alveolar tissue, and the evidence of early stages was all to the effect that they had originated from alveolar elements, like those induced in adults by methylcholanthrene (11). None had any direct connection with a bronchiole. But needless to say, as an adenoma grows large it must often fill the space into which a bronchiole opens and may even project into its lumen. The cells of the tumor of Fig. 18, in which this was the case, were not joined to those lining the bronchiole, yet a union might on occasion take place secondarily since experiment has shown that elements as widely

different as those of regenerating epidermis and of a carcinoma of prostatic origin can join in a layer (19). Furthermore the cells of pulmonary adenomas in adults sometimes resemble the epithelial elements lining the bronchioles (Fig. 17). It seems likely that these phenomena have provided the grounds for the belief that the growths often take origin from the bronchiolar lining,—the more so as the evidence on this point has been mostly obtained through studies of large, well established growths under conditions complicated by bacterial infection, atelectasis, and consolidation. If adenomas do arise now and again from the epithelium of the bronchial tree the occurrence must be highly exceptional.

The derivation of the cells lining the alveolar wall has long been the subject of controversy, some investigators holding that they are epidermal in nature and others that they are mesodermal (20). The changes taking place secondarily in the adenomas developing in the young of urethanized mothers provide evidence in this matter. Though the growths originated from the alveolar wall and had no direct connection with the bronchiolar epithelium, their cells not infrequently took on a resemblance to the latter within 60 to 70 days (Fig. 20). Occasionally in the growths of the mothers the resemblance was absolute (Fig. 17). Recently one of us (21) has found that the cells of the alveoli formed after the transplantation of fragments of mouse embryo lung to adult hosts may undergo metaplasia to stratified squamous epithelium if exposed to methylcholanthrene. The change may occur at spots distant from bronchiolar epithelium, as Passey has noted in rat lungs chronically inflamed by bacterial infection (22). These facts, taken together, make plain that the cells of the alveolar wall are pluripotential despite their specialization, and that they are epithelial in character. The hypothesis might be put forward as alternative that bronchiolar elements lie scattered amongst the alveolar ones, all undiscerned, and that the adenoma cells and squamous epithelium derive from these. But if this is the case why do not adenomas arise often from the bronchiolar lining instead of rarely if at all?

Certain workers with the spontaneous adenomas of adult mice and those induced with urethane believe that both arise on the basis of inflammatory lesions. If this were the fact the tumors occurring in the young of urethanized females could have resulted from neoplastic changes taking place after birth, in animals living long enough; the 6 months' existence of Larsen's mice would have provided abundant opportunity for this sequence of events. The present findings exclude it. Neither in the embryos removed from females receiving urethane repeatedly nor in their recently born offspring did any pulmonary inflammation occur which might have provided a basis for later adenomatous change; and no reactive proliferation or accumulation of lymphocytes or macrophages took place about the growths in the young animals (Figs. 1-14). The conclusion seems warranted that the neoplastic changes which found expression

in the adenomas of sucklings were not secondary to inflammation but primary in origin.

The action of urethane to elicit tumors has excited much comment, for it has appeared peculiar in several respects. The simplicity of the substance as compared with most oncogens has seemed to set it apart,—though subcutaneous injections of sugar cause sarcomas to arise in the mouse, and hydrochloric acid thus introduced will do so in rats. The finding has also seemed remarkable that urethane induces no growths except pulmonary adenomas,—though ergot gives rise to neurofibromas only, and Scharlach R to hepatomas, examples which need no longer be cited, now that urethane has been shown to elicit hepatomas as well as adenomas (23). The fact that a single exposure to the substance suffices for neoplastic change has also aroused speculation,—although a single painting of mouse skin with methylcholanthrene results in cancer in some strains of animals. More extraordinary is the finding that pulmonary adenomas arise in the absence of any visible tissue damage. Grady and Stewart (11), noting this of the adenomas induced with methylcholanthrene, were led to ask whether the chronic “precancerous” tissue alterations, which so generally precede the occurrence of tumors as almost to enter into any definition of them, are really essential to their origin. The question is worth asking again.

There remains to consider, of the peculiarities of urethane, the rapidity with which it induces neoplastic change as indicated by the present work. Here a distinction must be made between the preliminaries to such change and its actual occurrence. Though the preliminaries frequently extend over a long time the change itself takes place rapidly, according to all observation with oncogenic agents; it is as if a trigger had been pulled. The generality of agents press gradually upon the trigger; only its eventual click is abrupt. Urethane would seem to pull it hard. But another possibility exists, that the substance merely stimulates the proliferation of cells already neoplastic. Several authors have thought that this is its mode of action. The occurrence of problematic cell clumps in our young control mice might be viewed as supporting such a conception, and the extraordinary rapidity with which adenomas arose in the animals urethanized *in utero* accords well with it. Observations on the point are under way.

According to an axiom now well authenticated through experiment, the greater the natural liability of mice to this tumor or that the more readily can the growth be elicited by the application of an oncogen. Everything that is known of the response of various breeds to urethane falls in with this generalization. Nettleship, Henshaw, and Meyer (3), who first demonstrated that the substance induces pulmonary adenomas, noted also that it elicited many more of the tumors in A mice, the breed most prone to them spontaneously, than it did in C3H animals. Cowen (24) obtained data in R III, CBA, and C57 mice

which accord with this finding as do also the present results with adults of the C strain. Henshaw and Meyer (7) believed that the adenomas began to form within 1 to 2 months after the injection of urethane into 6 to 8-weeks-old animals of the A strain.

From all this one might have inferred that adenomas could scarcely have developed in animals of the C strain only a few days old. But the conditions of test were exceptional. Urethane is so diffusible that it must have reached the embryos in quantity. It was given in the maximum amount tolerable and the exposure of the pulmonary tissue to it was intensive; mice have not previously been injected with it day after day. Furthermore the tumors arose under conditions making for their rapid enlargement; embryos and newborn animals are notably favorable hosts for implanted neoplasms (25), the growing organism providing in abundance the stroma and vascularization that tumors need. And there was a deeper reason why the adenomas appeared so soon and contained mitoses in profusion, namely the state of the cells engaged in producing them. They were already vigorously proliferating to form the lung when they came under the influence of urethane. That they were behaving in this way because of an innate urge has been decisively proven by Cohn and Murray for the chick embryo (26) and is sufficiently attested for the mouse by the continuing growth of fragments of embryo lung after implantation in adult hosts. The adenomatous change superimposed upon the cells such further activity as enabled them to multiply even more rapidly than their normal fellows,—an advantage which became increasingly manifest when the proliferative activity of the latter fell off after birth.

These findings bear on what is implicit in the neoplastic state. Widely various examples are on record of the extraneous stimulation of tumor cells; numerous substances have been found to promote the growth of tumors produced experimentally in animals and one often sees the phenomenon clinically, *e.g.*, after a growth becomes infected with pus-producing organisms, or when testosterone urges on the prostatic carcinomas of man. All such happenings are comprehensible because the agents urging the growths on stimulate normal tissues as well; they act as adjuvant influences merely, and when they are no longer present their influence lapses. The happenings after adenomatous change has taken place in the young of urethanized females stand in a different category. In their case stimulation resulting from neoplastic change is superimposed upon a proliferative activity natural to the cells of the very young organism. Yet again the relationship is not enduring. As the animal becomes mature and its normal pulmonary cells almost stop multiplying, the tumor cells cease to nearly the same extent. Now their only advantage is that which the neoplastic state itself brought with it, and under the circumstances obtaining in the adult animal this suffices for but the slowest proliferation. The tumor cells have remained susceptible to the normal ageing influences. No longer

active, they differentiate until they more or less closely resemble the normal bronchiolar epithelium.

These facts appear to provide a partial answer to the question whether the period in the life of the organism at which cells become tumor cells has any importance for the neoplastic process set up within them. The period has indeed an influence in the case of the growths under discussion. When adenomatous change takes place in the very young organism it increases the activity of cells already possessed of a natural tendency to divide; and for this reason, as well as because of their highly favorable environment, they proliferate more rapidly than the adenoma cells of adults. But the relationship which has this consequence is no more than additive; and the most effective of its factors, those due to youth, soon wane and are gone.

Although the urethane acted upon embryos and the resulting adenomas were perceptible within the first days after birth, this does not mean necessarily that the pulmonary cells became tumor cells *in utero*. The relative size of the growths in the 10-day- and 3-day-old animals, and the failure to find any with certainty in fetuses and animals just born prove that their formation took place almost wholly after birth. But it could scarcely have been otherwise. The longest interval from the first urethane injection to parturition was 11 days (Table 2), and during the first 2 of these the lungs had barely begun to form. Nevertheless when the animal was killed, 3 days after birth, or after 14 days in all, an adenoma of considerable size had developed (Figs. 4 and 5). In no other instance of the sort was the elapsed time so long. The interval before birth in a second 3-day animal with a smaller tumor (Figs. 6 and 7) was 8 days, the total elapsed time 11 days; and in a third mouse, with a still less developed growth (Figs. 9 and 10), 7 days and 10 days respectively. The length of postnatal life was constant in these instances, only the prenatal interval varied. Yet much cannot be made of this circumstance as bearing on the size of the tumors, since the urethane may not have brought about adenomatous change until it had been given several times.

While the evidence is strong that neoplastic conversion was consummated *in utero* the possibility still must be considered that the urethane merely rendered the young animal susceptible to the influence of some extraneous tumor-producing agent reaching it immediately after birth. One thinks of an agent like that responsible for mammary cancer in the mouse, which is passed on from mother to young in the milk. But the data of Table 3 provide no support to this conception, disclosing as they do no correlation whatever between the incidence of adenomas in mothers and their young. The results of four or six injections of urethane are especially noteworthy in this regard; though the mother animals in some of these instances developed multiple adenomas, their young showed almost none. This is the more significant in view of the cumulative effect of urethane to elicit adenomas in adult animals, the rapidity with which these de-

clared themselves in the gross in young animals; and the fact that the offspring of females receiving urethane only once often showed them (Table 3). It would appear that, for reasons unknown, potentialities for the growths were slight or lacking in the embryos of those mice that happened to be many times injected. The existence of small islands of cells resembling the first stages of adenomas, in some of the control baby mice but not in others, may have a bearing on why the growths appeared in only a proportion of the individuals exposed to urethane instead of in all of them.

As already stated, the observations here recorded were made, like others from this laboratory, to learn how soon in the life of the organism cells possess the ability to become tumor cells. Growths arise so rapidly and in such diversity from mouse embryo tissues, after transplantation with methylcholanthrene to adult mice, as to make it well nigh impossible to suppose that extraneous actuators resembling those now known (the tumor-producing viruses), and entering after birth, are responsible for the generality of neoplastic changes. The facts all have indicated that the cells of the embryo possess the ability to become tumor cells (1). The present demonstration that the injection of urethane into pregnant mice causes adenomatous changes to come about so quickly in the young they carry that tumors are perceptible almost at once after birth makes it difficult to avoid the conclusion that the pulmonary cells of mice in mid-fetal development are capable of being neoplastic. This capability would appear to exist earlier in the life of the organism than the one for mammary tumors, which is conferred at the first nursing. Evidently each type of neoplasm must be studied for itself in such relation.

The relative share of the intrinsic and the environmental in neoplastic change is amongst the most deep-going questions in cancer research. Yet only the environmental factors have been inquired into with particularity thus far for the good reason that practically all observations have been made of necessity upon growths coming to attention after birth,—upon those developing in organisms which had passed out from the protection of the uterus into a world in which they were beset by oncogenic agents. Even the hereditary and familial tendencies to tumors, manifest in inbred strains of mice, have been perceptible only in terms of postnatal happenings. Gideon Wells could find but 66 valid instances of tumors in newborn infants, none of them carcinomas (27),—a negligible number, it may be remarked, in comparison with the instances of disease due to viruses reaching human embryos *in utero*. True, hydatidiform moles and chorio-epitheliomas are due to neoplastic changes in a tissue of embryonic origin: but the chorionic cells are not shielded by the natural contrivances whereby the embryo itself is protected; on the contrary they are even more exposed to environmental factors than the majority of the cells of the mother, bathed as they are directly in her blood. The cells composing embryonic rests and the frank embryomas and teratomas from which tumors

take off in later life are not only subject to the same environmental influences as the cells of the host but are exposed to special hazards, often becoming the seat of pathological disturbances which may render them exceptionally liable to neoplastic change.

SUMMARY AND CONCLUSIONS

The observation that adenomas develop very rapidly in the pulmonary tissue of mouse embryos, implanted together with methylcholanthrene in adult animals, has led to tests of the neoplastic potentialities of this tissue *in utero*. C strain females in the latter half of pregnancy were injected with urethane and the lungs of their young were searched for adenomas. None could be perceived with certainty in embryos at term or in mice just born, but they were several times found 3 days after birth and they were frequent and much larger in 10-day-old animals. The controls showed none. After 60 to 70 days they were often visible in the gross. Corroboratory findings were obtained in A mice. No parallelism could be perceived in the incidence of the tumors in mothers and offspring.

The adenomas arose from tissue devoid of any sign of preliminary local disturbance. Mitoses were abundant in them and they grew rapidly for a while, but within 2 months cell division had almost ceased. By this time however many of the neoplasms were as big as any adenomas in the urethanized mother animals and in some instances twice as big. While growing fast they underwent little differentiation, but this took place when proliferation slowed and in consequence the tumors came to have the morphology of the spontaneous and induced adenomas of adults.

The neoplastic cells were derived from alveolar elements, yet in proportion as differentiation of them occurred they came to resemble the epithelial cells lining the small bronchioles. Occasionally the resemblance to bronchial epithelium was complete, save that the cytoplasm of the tumor cells was slightly basophilic.

The following conclusions seem justified:—

1. The injection of urethane into pregnant female mice of the C strain frequently initiates the development forthwith of pulmonary adenomas in the young she is carrying.
2. Some of the pulmonary cells of mouse embryos well along toward term possess the ability to be neoplastic.
3. The adenomatous change finds swift expression in young creatures because of conditions implicit in their youth. The rapid proliferation of the tumor cells is almost entirely due to these conditions, not to the neoplastic state as such.
4. Adenomatous change prior to birth is intrinsically the same process as that occurring in the adult creature.

5. The adenomatous state does not prevent the cells of young mice from undergoing the maturation that takes place in normal elements of the same sort as the organism grows older. Though the proliferative activity natural to youth and the unnatural activity consequent on neoplastic change are summated in the young organism, they still are separable.

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EXPLANATION OF PLATES

The photographs were made by Mr. J. A. Carlile.

All of the sections were stained with eosin and methylene blue.

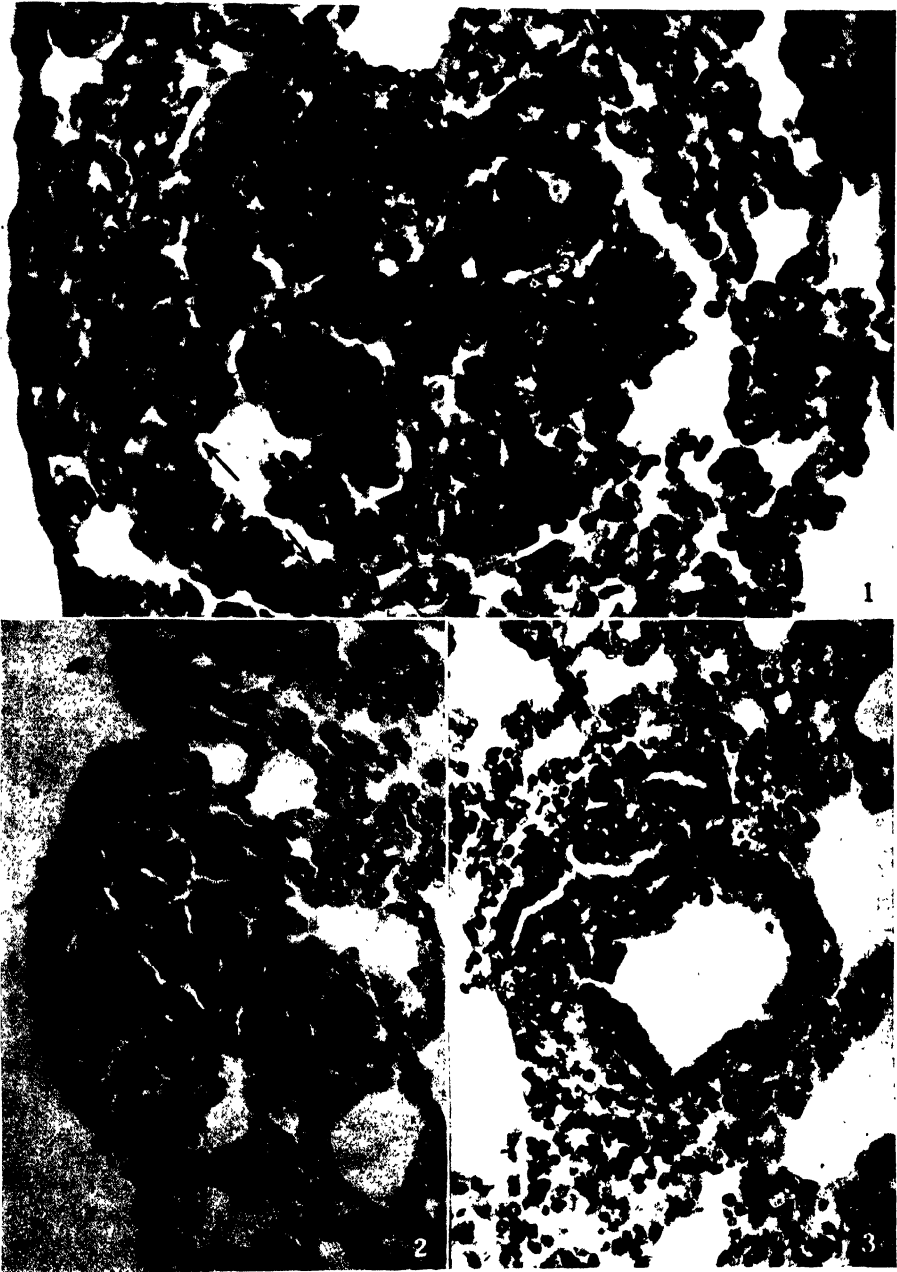
PLATE 24

FIG. 1. Adenoma in a 10-day-old mouse from a mother that had received six injections of urethane, the last within 24 hours of parturition (mother IV of Table 1). The growth lies near the lung surface and has compressed the parenchyma between it and the pleura,—seen on the left. Its cells are more basophilic than those of the parenchyma about them and they nearly all have large, round vesicular nuclei. The arrows point to two of the many mitotic figures. $\times 505$.

For other growths in animals of the same litter see Figs. 3, 9, and 10.

FIG. 2. Adenoma in a 10-day-old animal from another mother getting six injections, the last of them 3 days before parturition (mother VIII of Table 1). The markedly basophilic growth protrudes on the pleural surface. It has filled several alveoli with compact masses of cells but its central portion is adenomatous. Other sections showed mitoses to be fairly numerous in it. $\times 294$.

FIG. 3. Adenoma in a 10-day-old sibling of the mouse furnishing Fig. 1. As in the case of the other early tumors there is no reactive proliferation or cellular accumulation about the growth. It looks as if it had originated from the epithelium of the bronchial tree, but serial sections showed it to be isolated amidst parenchyma. $\times 321$.



(Smith and Rous: Neoplastic potentialities of mouse embryo tissues. IV)

PLATE 25

FIG. 4. Adenoma in a 3-day-old mouse born 7 days after the last of four urethane injections into the mother (mother IX of Table 2); total period since the first injection, 14 days. At the level here pictured there appear to be three separate islands of neoplastic proliferation, but they were parts of a single growth. It lay in the parenchyma far from any bronchiole and stained a much deeper blue than the epithelium lining the latter. $\times 186$.

FIG. 5. Higher magnification of the same tumor at another level, -to show its "glandular" arrangement and cuboidal cells. $\times 401$.

FIG. 6. Subpleural adenoma in a 3-day-old mouse born 2 days after the last of six injections of urethane into the mother (mother X of Table 2); total period since the first injection, 11 days. The growth is discrete. $\times 250$.

FIG. 7. The same tumor at higher magnification. It is of typically adenomatous character. There is no cellular reaction about it. $\times 569$.

FIG. 8. Clump of deeply basophilic cells with vesicular nuclei; lung of a 3-day-old litter mate of the animals providing the growths of Figs. 1, 3, 9, and 10. Similar clumps of cells have occasionally been seen in normal C strain mice 10 days old. $\times 569$.



(Smith and Rous: Neoplastic potentialities of mouse embryo tissues IV)

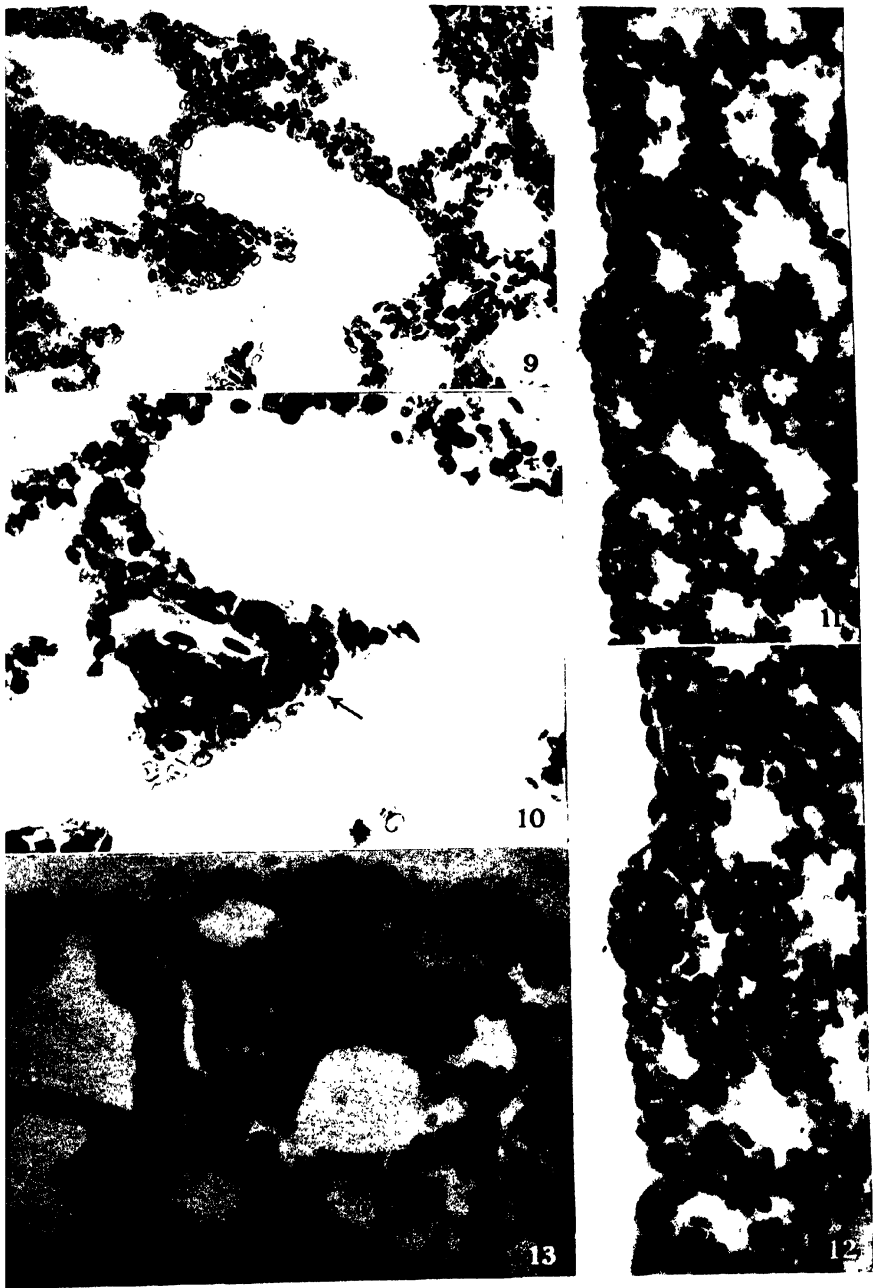
PLATE 26

FIG. 9. Adenoma in a 3-day-old litter mate of the animals furnishing Figs. 1, 3, and 8. The growth lies in the parenchyma and is of the compact type. It stained bright blue against the general pink. $\times 277$.

FIG. 10. Section through the edge of the growth of Fig. 9, where it covers part of an alveolar wall with a single layer of cells. The arrow points toward a mitosis; there were three in the tiny growth. $\times 527$.

FIGS. 11 and 12. Adenoma (?) in the lung of an embryo near term. The mother had received six injections of urethane, the last one 24 hours before the embryo was procured. The growth bulges on the pleural surface, appears to have some acinar arrangement, and its cells occupy part of a neighboring alveolus. The embryonic state of the pulmonary tissue is evident. Fig. 11, $\times 280$. Fig. 12, $\times 449$.

FIG. 13. Adenoma of the glandular type in a 10 day-old mouse from a mother of the A strain which had received three injections of urethane, the last within 24 hours of parturition; total interval since the first injection, 14 days. $\times 449$.



(Smith and Rous: Neoplastic potentialities of mouse embryo tissues. IV)

PLATE 27

FIG. 14. Another "glandular" adenoma from the same animal; there were only the two growths. The tumor is markedly basophilic, as in the previous instance. Some atelectasis is present owing to delay in fixation of the lung after it had been removed from the animal. $\times 422$.

FIG. 15. Compact adenoma in a 10-day-old mouse from another female of the A strain injected thrice with urethane, the last time within 24 hours of parturition. The growth shows the usual basophilia. The arrow indicates a mitotic figure. $\times 422$.

FIG. 16. Mooring clump with membrane attached near by; from a day-old mouse of a C strain mother. Serial sections showed the clump to be spindle-shaped and almost $300\ \mu$ long. The arrow points to a mitosis. $\times 422$.

FIG. 17. Peripheral zone of an adenoma, with part of an adjacent bronchiole, in a mouse killed 70 days after birth of her offspring. She had received three injections of urethane during pregnancy.

The growth differs considerably from those in sucklings. Interspersed among cells with round, vesicular nuclei, such as make up the tumors in these latter, are many with nuclei that are oval or oblong and almost pyknotic, often relatively large and sometimes with more cytoplasm about them than ordinary. In one spot near the lower edge of the picture nuclei of this sort lie with their long axes parallel, and the cells appear cylindrical, as if from lateral compression. The epithelium of the bronchiole has precisely the same general morphology as the component elements of the tumor except that some of its cells are more cylindrical. $\times 278$.

FIG. 18. Low magnification of the same tumor, to show where it has filled a space into which the bronchiole opens. Though its cells closely resemble the epithelium of this latter the two are not joined. At the center of the growth, where degeneration was under way, the cytoplasm had a blue cast on staining, but at the periphery it was almost as pink as the bronchiolar epithelium. $\times 136$.

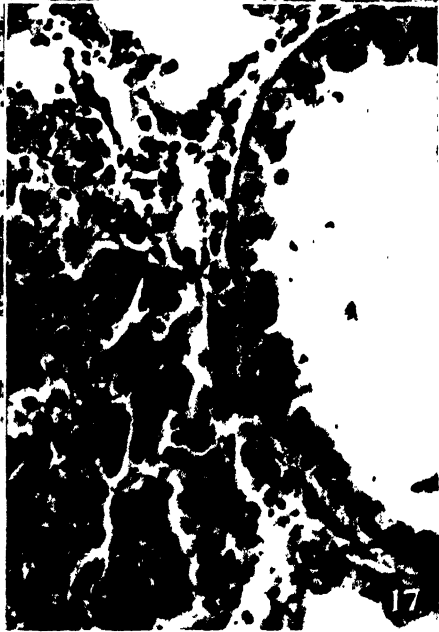
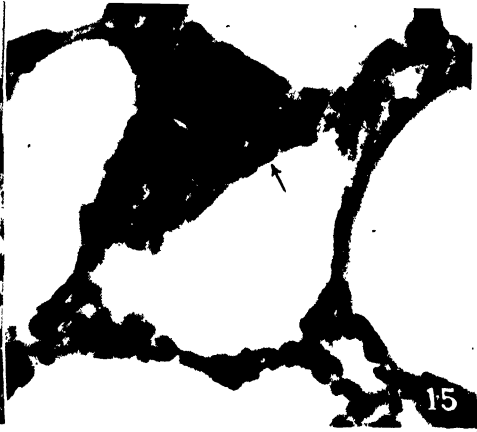


PLATE 28

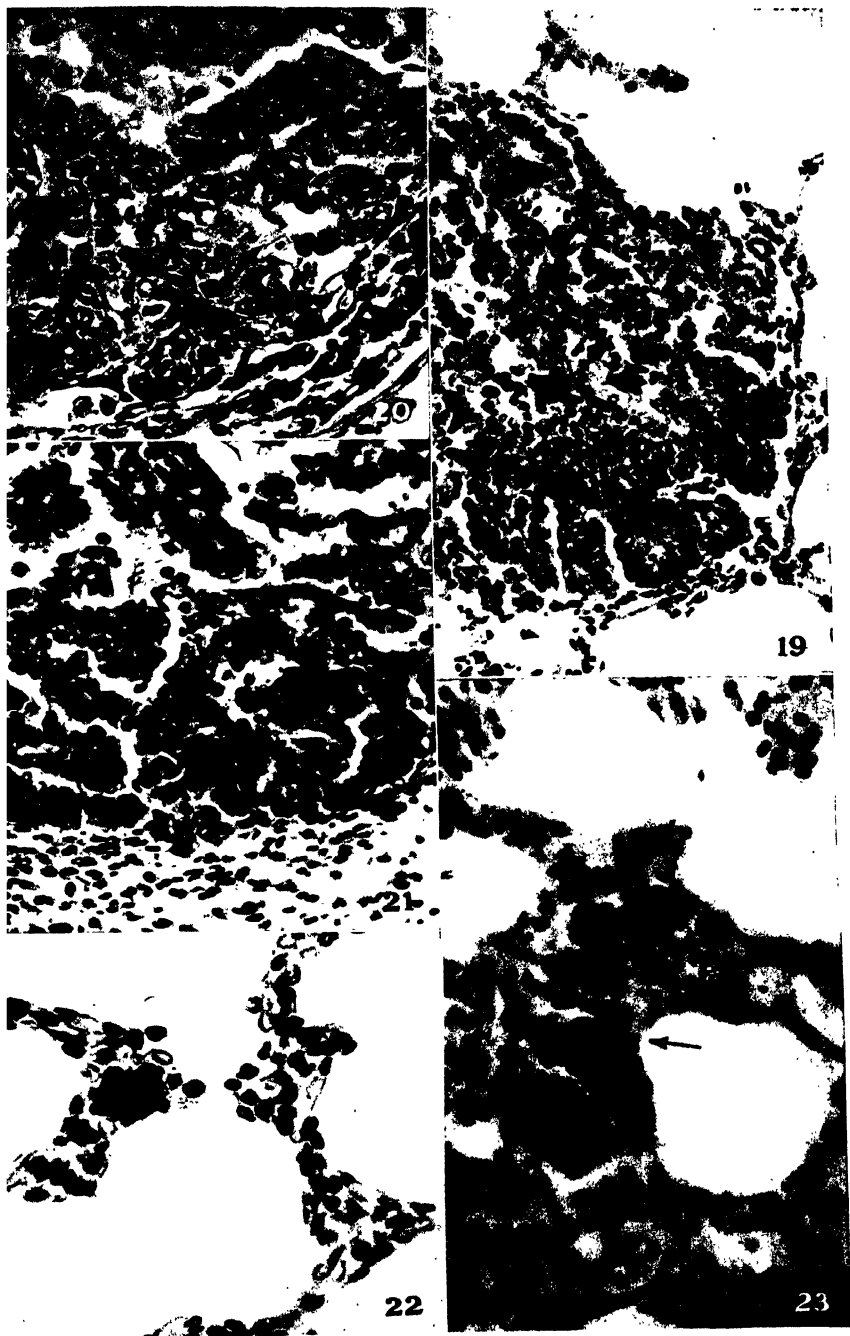
FIG. 19. Another adenoma from the same animal as that of Figs. 17 and 18 and with the same cytological features. It was not directly connected with any bronchiole. $\times 243$.

FIG. 20. To show the "bronchiolar" character of the cells of an adenoma found in an animal 70 days after birth from a urethanized mother (mother G of Table 3 and 4). Though unusually large (1.5 mm. in diameter) the growth was devoid of mitotic figures. The tissue next its border is compressed. $\times 449$.

FIG. 21. Edge of another exceptionally large adenoma (2 mm. across) in a 70-day-old mouse from a urethanized mother (mother A of Table 3 and 4). The growth is markedly basophilic and differentiation is not as far advanced as in the tumor of Fig. 20 yet many dark-staining nuclei are present, of the sort found in mature adenomas. Mitotic figures were rare (Table 4). $\times 277$.

FIG. 22. Clump of cells in an alveolar wall of a normal 3-day-old mouse of the C strain. The cells are notably basophilic, as in the case of adenomas, but the nuclei were smaller than in these latter and were not vesicular. $\times 527$.

FIG. 23. Dubious growth in another 3-day-old control of the C strain. The cells are basophilic and at one spot they lie in two parallel rows (arrow), but many of the nuclei are oblong and they are unusually small. $\times 449$.



(Smith and Rous: Neoplastic potentialities of mouse embryo tissues. IV).

AFFERENT FIBERS IN MUSCLE NERVES

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The present investigation was undertaken to obtain classification according to diameter of the myelinated afferent fibers distributed to a number of individual muscles of the hind-limb, the primary purpose being to determine the pattern of afferent nerve fiber supply to limb muscle, and the degree of conformity, among the individual nerves, to that pattern. To this end it was important to study nerves in series as homogeneous as possible. Accordingly, extensive ventral root section was practiced to permit the subsequent removal, from each preparation, of a considerable number of individual "demotored" muscle nerves, which then were prepared in a batch for microscopical examination.

Methods

For one preparation (Cat I) the 5th, 6th, 7th, 8th, and 9th post-thoracic ventral roots on the right side were sectioned intradurally, a piece 5 to 10 mm. in length being removed in each instance. For the other preparation (Cat II), the 4th, 5th, 6th, 7th, 8th, 9th, and 10th post-thoracic ventral roots of the right side were sectioned in the same manner. The operations were performed aseptically with the animals anaesthetized by nembutal. During the degeneration period—122 days for Cat I, 103 for Cat II—repeated examination revealed no apparent loss of cutaneous sensibility. Paralysis was incomplete at the hip in Cat I, reflecting the integrity of the 4th post-thoracic ventral root. Only nerves receiving contributions from the 6th, 7th, and 8th post-thoracic segments were removed from this preparation. The right leg of Cat II was completely paralyzed. At the time of sacrifice there had been no restoration in motor function.

Following the period of degeneration the nerves were exposed, identified and excised. Each nerve was splinted on a labelled card and transferred to osmic acid (0.5 g. with 0.5 g. NaCl in 100 cc. distilled water) for about 40 hours, after which the nerves were washed, dehydrated in small steps through ascending grades of alcohol, imbedded in paraffin, and sectioned at 5 to 8 μ thickness. Every attempt was made to obtain sections from the mid-stretch of nerve to avoid handling distortion, and at a uniform distance from the point of junction with muscle to have the various nerves roughly comparable with respect to afferent fiber branching (cf. 2). The spinal cords were examined at autopsy to confirm the root sections.

Sections selected for fiber measurement were projected on to white paper at a magnification that permitted an image of the entire nerve to be formed. The visible fibers were drawn without undue precision, inasmuch as the maps so constructed were to

serve only for recording the measurements made by use of the ocular micrometer. Any fibers missing from the maps were entered, and spurious entries removed, at the time of measurement. Available equipment included a Zeiss $\times 100$ objective and a Leitz micrometer ocular accurately calibrated to read one division per micron. These were used for all measurements.

Sources of error in measurements of nerve fibers frequently have been discussed (1, 4, 10, 11). A number of the recognized factors introducing error apply to the present measurements; others do not. Outline distortion was considerable in the nerves from Cat I and minimal in the nerves of Cat II, making the latter the more

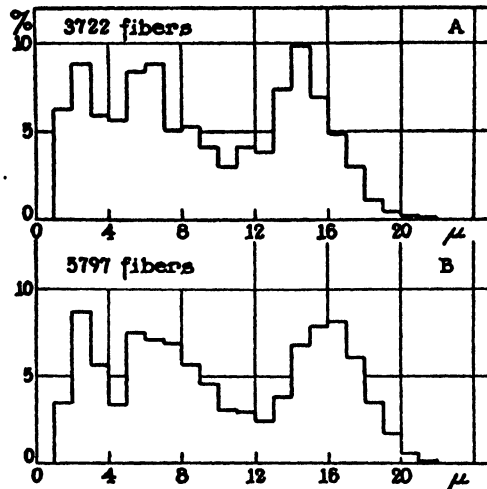


FIG. 1. Fiber caliber spectra of "demotored" muscle nerves. For each plot are enumerated all the afferent fibers measured in the muscle nerves of a single preparation, 3722 fibers in 12 nerves of Cat I, 5797 fibers in 16 nerves of Cat II. The nerve fiber diameters in micra are plotted against numerical distribution in percentage of total afferent fibers.

reliable objects for study. Variation in diameter is reason for concern in all measurements of nerve fibers. Cat I weighed 3.25 kilo at operation, Cat II, 4.0 kilo. There might be cause here for the fibers in the nerves of Cat I being generally of less diameter, but greater shrinkage is undoubtedly the prime factor. Judging by comparison with other published accounts (1, 11) of fiber diameter in osmicated and fresh nerve, shrinkage of fibers from Cat II was minimal. There is no error of sampling, for all the remaining myelinated fibers in each nerve were counted and measured. Variation in magnification was eliminated. There is no error arising from tracing the fibers. The fibers in all of the nerves from Cat I were counted and measured by one of the authors, those in the nerves of Cat II by the other. This served to preserve uniformity of treatment within each series of nerves. Several recounts by the same observer after three months, and cross-checks by the other observer, indicate a reasonable agreement, differences greater than 0.5μ being unusual. Two measurements of

each fiber were made, the mean diameter being entered on the map in the circle representing that fiber. Reference to Figure 1, in which are plotted according to diameter, in A, all of the measured fibers of Cat I and, in B, all of the measured fibers of Cat II (the measurements for A being made by one author, the measurements for B by the other), shows that the general character of the muscle afferent fiber caliber spectra is similar for the two series studied on a largely independent basis. The measurements of fibers in nerves obtained from Cat II are to be preferred for the reasons stated.

RESULTS

The general pattern of distribution according to diameter of afferent fibers to muscle may be seen in Figure 1A and B. For Figure 1A are enumerated all of the afferent fibers in Cat I distributed to:

semitendinosus	tibialis posterior
semimembranosus	flexor longus digitorum medialis*
gastrocnemius lateralis	flexor longus digitorum lateralis*
gastrocnemius medialis	tibialis anterior
soleus	extensor longus digitorum
plantaris	peroneus longus

In the fiber diameter plot of Figure 1B are represented all of the afferent fibers in Cat II distributed to the following muscles:

rectus femoris	soleus
vastus lateralis	plantaris
sartorius	tibialis posterior
crureus	flexor longus digitorum medialis*
semitendinosus	flexor longus digitorum lateralis*
biceps femoris posterior†	tibialis anterior
gastrocnemius lateralis	extensor longus digitorum
gastrocnemius medialis	peroneus longus

The most obvious feature of the afferent fiber distribution plots is the occurrence of three peaks of numerical preponderance, centered at $15\text{--}17\mu$, $6\text{--}7\mu$, and at 3μ . In the individual nerves the distinction between the group of largest fibers and the remainder is usually well marked by a numerical deficit of fibers measuring $12\text{--}13\mu$ in diameter (Figs. 2 and 3). The peaks contributed by the medium and small-sized fibers are separated by a minimum centered at 5μ . This latter minimum occasionally is not well marked, but the significance of this fact may be operational rather than real, for, in general, the artifacts of

* This designation has been chosen for the two heads of flexor longus digitorum, otherwise known by the adjectival designation "tibialis" and "fibularis" or, as in man, by the distinct titles flexor longus hallucis and flexor longus digitorum.

† The long nerve to the inferior portion of biceps femoris posterior. This nerve is particularly useful for reflex studies.

preparation and the vagaries of measurement must be expected to obscure significant groupings rather than to introduce artifactual peaks of preponderance.

The existence of three major groupings is supported by functional evidence. According to studies on the reflex results of stimulating muscle afferent fibers, the largest fibers, known collectively as Group I fibers (6), form direct connections with motoneurons to provide a monosynaptic reflex discharge that represents the myotatic reflex. The medium and small fibers, those allocated to Group II and Group III, connect, on the ipsilateral side, through internuncial relays to motoneurons predominantly of flexor muscles. Stimulation of these latter groups results in two successive flexor reflex discharges that differ greatly in afferent threshold and central latency. A cutaneous nerve on stimulation secures a qualitatively similar result, there being again two comparable flexor reflex discharges of differing afferent threshold and latency. From the viewpoint of reflex effect then, the groups of medium and small afferent fibers in muscle nerve, as far as one can tell, are comparable to the two prominent fiber aggregations in cutaneous nerves. Furthermore, a comparison of the muscle afferent fiber distribution plots here presented, with the fiber plots of cutaneous nerves published by Ranson, Droegemueller Davenport and Fisher (9), and by Gasser and Grundfest (3) reveals, between demotored muscle nerves and cutaneous nerves, a rather remarkable similarity in the fiber size range from approximately 13μ down to the smallest myelinated fibers. This similarity is the more remarkable when consideration is accorded the different modalities of sensation served by the two classes of fibers, cutaneous and muscular in origin. Indeed, the parallelism is such as to raise the possibility that fiber size is related more to the requirements of reflex performance than to those of the modalities of sensation.

Afferent Fiber Distribution Pattern of Individual Muscle Nerves

Comparison of Figures 2 and 3 with Figure 1 indicates that the distribution of fibers according to diameter, as revealed by the pooled counts of hind-limb nerves, is maintained reasonably constant among the individual muscle nerves. In each instance the three aggregations are discernible. There are, however, certain individual variations that appear worthy of note. One of these is the distinction between the afferent fibers supplying red and pale muscle. Hay (5), comparing the afferent fibers to the red soleus and pale semimembranosus in the rabbit (in one preparation), noted that the largest afferent fiber to soleus lay in the $15\text{--}15.5\mu$ category of diameter, while that to semimembranosus lay in the $17.5\text{--}18\mu$ category, the latter muscle possessing 43 fibers greater in diameter than 15μ .*

* It is impossible to give the percentage of total represented by the 43 fibers, for Hay did not enumerate the fibers of less than 10μ diameter.

Eccles and Sherrington (2) mention, in the course of their observations on motor units, finding that the largest afferent fibers of soleus nerve were about 13μ , whereas those of gastrocnemius ran to $18-19\mu$. Since, as may readily be seen in Figures 2, 3, and 4, redness or paleness of muscle is not the only consideration relating to size of its afferent fibers, it would seem most correct to compare, as Eccles and Sherrington did, the red and pale heads of a given muscle, although in fact there exists a sharp distinction between the upper limit of afferent fiber size to red muscle heads and to all the other extensor muscles of the series (Fig. 4). The present preparations offer three instances in which comparison of red and pale muscle afferent innervation may be made, the muscle pairs being soleus and gastrocnemius, or crureus and vastus. In one instance the largest fiber to soleus was 17μ , the largest to gastrocnemius 19μ , and 4.65 per cent of the total fiber population to the latter muscle consisted of fibers greater than any to soleus. In another the largest soleus fiber was 17.5μ , that to gastrocnemius 21μ , with gastrocnemius receiving 10.25 per cent of its fibers in diameter categories greater than any supplying soleus. Finally crureus possessed as its largest fiber one of 17.5μ , vastus one of 21.5μ with 14.5 per cent of its fibers greater than the largest to crureus. The present observations, therefore, are in agreement with the passing references of the earlier authors.

Inasmuch as muscles other than the red variety fail to receive afferent fibers of the largest diameters, although in every case a distinct grouping in the range above 12μ is present (Figs. 2 and 3), it is of interest to consider briefly the function of the muscles that exhibit the deficiency. In addition to the red soleus, among the muscles supplied by the popliteal nerve, tibialis posterior is such a muscle. Indeed, tibialis posterior is an interesting exception among the popliteal group of muscles for other reasons. In contraction (cat) it is seen to invert the foot without contributing to flexion or extension, all the other muscles of the group acting for physiological extension. It is neither excited nor inhibited in the course of a flexion reflex (12). Finally, in study of the monosynaptic reflex actions of tibialis posterior afferent fibers (7), it has been found that these fibers, on stimulation, provoke a sizeable monosynaptic reflex discharge in the seventh post-thoracic ventral root, and yet they wield no direct influence, excitatory or inhibitory, upon motoneurons of other muscles of the ankle—this in sharp contradistinction to the behavior of ankle flexors and extensors that are linked together by facilitatory or inhibitory interconnection according to the requirements of reciprocal innervation. In turn, activity engendered in the large afferent fibers of the other ankle muscles does not, by direct connection, influence a monosynaptic reflex of tibialis posterior. In short, tibialis posterior possesses a monosynaptic, or myotatic, reflex of its own, but does not participate, with the flexors and extensors, in the myotatic unit of the ankle.

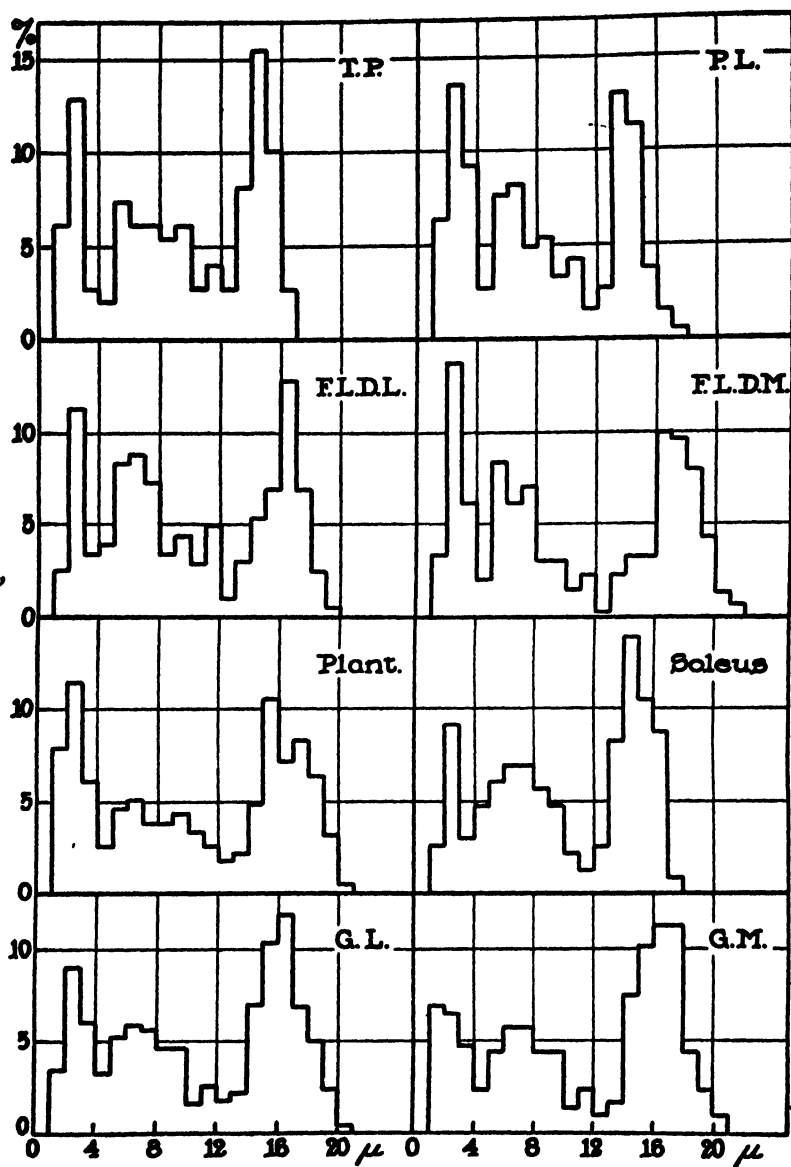


FIG. 2. Fiber caliber spectra of individual "demotored" muscle nerves of Cat II. The plots are constructed as for Fig. 1. The individual muscle nerves are identified by abbreviations identifiable by reference to the table of muscle nerves in the text.

Turning now to a more general discussion of the afferent fibers of individual muscle nerves, it will be noted, in examination of Figures 2 and 3, that the

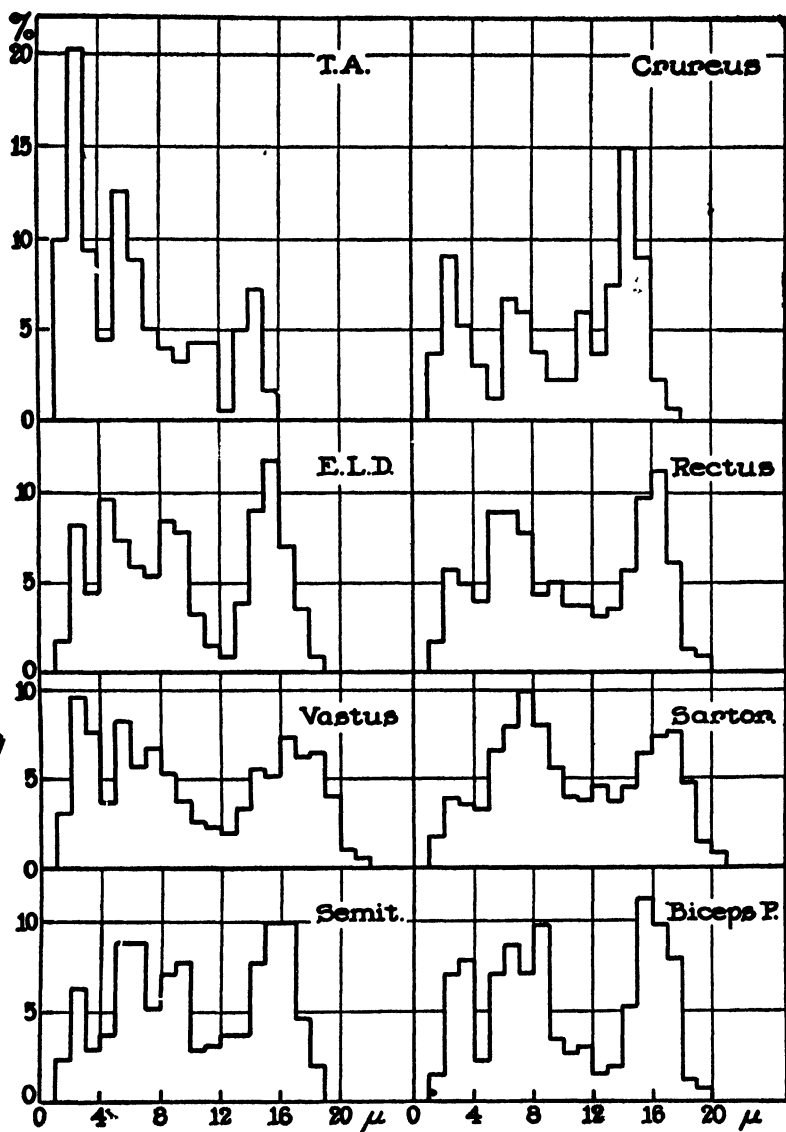


FIG. 3. Fiber caliber spectra of individual "demotored" muscle nerves of Cat IZ. This figure is a continuation of Fig. 2, and is constructed in identical fashion.

largest afferent fibers of the flexor muscles appear not to reach in caliber those of their extensor antagonists, excluding, of course, the red muscles.

In Figure 4 is presented a graph designed specifically to illustrate the fore-

going propositions relative to the variation, in upper limit of diameter, of the afferent fibers in the nerves to individual muscles. Each point in Figure 4

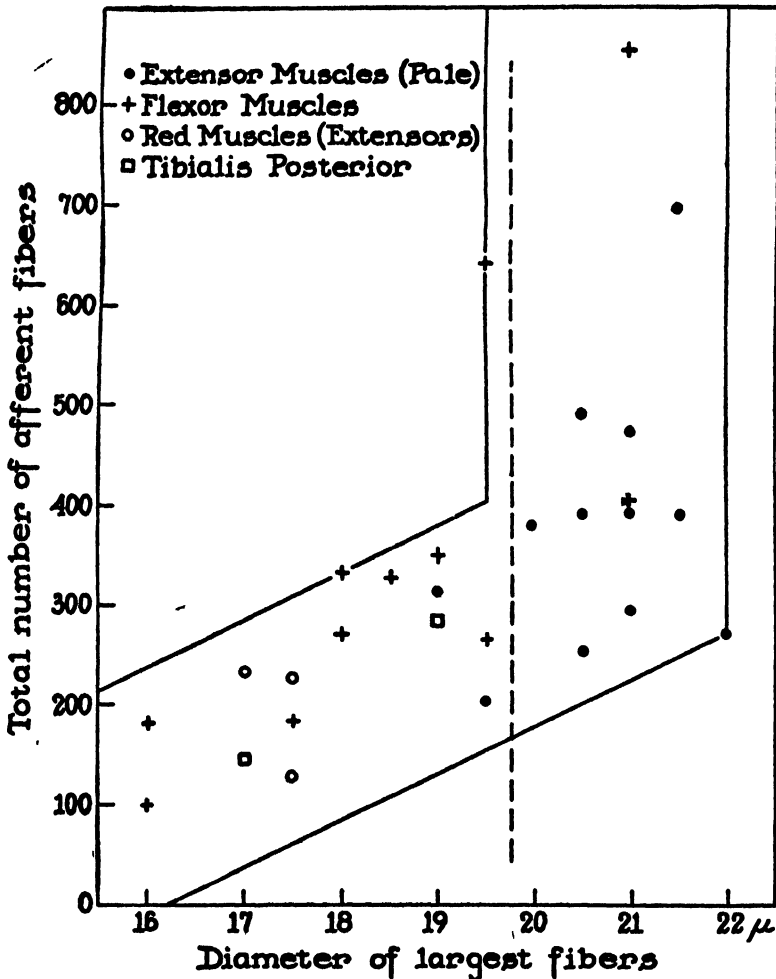


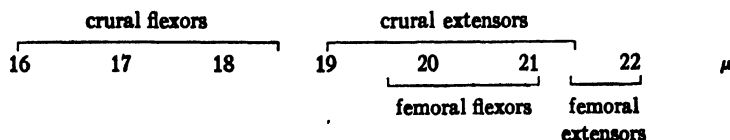
FIG. 4. Relation between the total number of afferent fibers supplying a given muscle, and the largest afferent fibers supplying that muscle. Each point represents an individual muscle nerve. The action and type of the individual muscles supplied are identified by the distinguishing symbols.

represents an individual muscle, and is located on the ordinates according to the total number of afferent fibers distributed to the muscle, and on the abscissae according to the largest afferent fibers among the total. Considering

together all the muscle nerves, regardless of action or type of muscle supplied by them individually, there appears, on examination of Figure 4, to exist, between the total number of afferent fibers to a muscle and the size of the largest afferent fibers to that muscle, a direct relation that holds until the upper limit of nerve fiber caliber ($20.5\mu \pm 1.5\mu$) is reached. *Ceteris paribus*, this could mean merely that a large collection of nerve fibers of a given functional affinity has a better chance than a small one of containing members at the extreme ends of a normal distribution.

On the other hand, when the types and actions of the individual muscles, as distinguished by the identifying symbols in Figure 4, are considered, it is immediately obvious, for instance, how wide is the difference between the largest fibers distributed to the red muscles (circles) and the largest fibers distributed to all of the other extensor muscles (dots). Furthermore, if an arbitrary division is made—as represented by the broken line in Figure 4—between the muscles receiving afferent fibers greater than 19.5μ and those that do not, it is found that the former group contains ten extensors and but two flexors, whereas the latter group contains nine flexors and but two extensors, these last naturally in addition to the previously discussed three red muscles. Eliminating the red muscles and tibialis posterior from further discussion, it is apparent that most extensors possess larger afferent fibers than do most flexors.

Even greater distinctions between the upper limit of afferent fiber sizes to flexor and extensor muscles appear if the nerves of femoral muscles and crural muscles are considered separately. Justification for this procedure may be found in the facts that all femoral flexors are supplied by afferent fibers greater in diameter than any to crural flexors and, although the spread is not nearly so great, the largest fibers to femoral extensors are as large as, or larger than, any seen in the nerves of crural extensors. Thus, in so far as it is permissible to generalize from the present series, it would appear that the more proximally situated muscles receive larger afferent fibers than do the more distally situated muscles, and of these two groups of muscles, femoral and crural, in each case none of the flexors receives afferent fibers of a caliber equalling that of the largest afferent fibers to any of the extensors. The range of maximal afferent fiber sizes to muscles, femoral and crural, flexor and extensor may be summarized to advantage in the following scheme:



SUMMARY

A study of the afferent nerve fiber supply to a number of muscles of the hind-limb has led to the following conclusions, some old, some new.

All sizes of myelinated fibers are represented among the afferent nerve fibers of the muscles named, the upper limit of fiber size, however, varying somewhat from one nerve to another.

The fibers, in categories of diameter, are clustered about three peaks of numerical preponderance.

The characteristic pattern of the afferent fiber caliber spectrum is repeated, with minor variation, in each of the individual muscle nerves.

Concerning the upper limit in diameter of afferent fibers distributed to hind-limb muscles, the present observations indicate that:

1. The more proximal muscles (femoral group) receive larger afferent fibers than do the more distal muscles (crural group).
2. Extensor muscles (pale) receive larger fibers than do their flexor antagonists.
3. Many (4.65—14.5 per cent of total) of the afferent fibers to pale heads of an extensor muscle are larger than any to the red fraction of the same muscle.

The paper, "Calibre spectra of motor and sensory nerve fibres to flexor and extensor muscles," by B. Rexed and P. O. Therman (*J. Neurophysiol.*, 1948, 11: 133-139) appeared as the present manuscript was in course of publication. Rexed and Therman describe the three peaks of numerical preponderance in caliber spectra of demotored muscle nerves. The present paper stands in confirmation of their finding. Difference in the two accounts, as to location of the peaks on the scale of diameters, arises from the evidently not inconsiderable shrinkage of fibers during preparation by the modified Alzheimer-Mann-Häggqvist method. Consonant with this interpretation one finds little discrepancy at the small fiber end of the spectrum, considerable at the large fiber end.

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ANALYSIS OF FORELIMB-HINDLIMB REFLEX ACTIVITY IN ACUTELY DECAPITATE CATS*

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This paper is concerned with an analysis of long spinal reflex action. The point of departure for experiment was the knowledge that afferent volleys stimulated in the brachial plexus caused inhibition of certain motoneurons of the lumbar enlargement, the latency of the effect being such as to imply mediation by the simplest possible cervico-lumbar propriospinal paths, and that, after a further delay, the brachial plexus volleys provoked a discharge of interneurons in the ventral horns of the lumbar enlargement, accompanied by facilitation of motoneurons in that region (5). Which motoneurons of the hindlimb were inhibited, which facilitated, was not known; neither were the properties of the executant afferent fibers, nor the receptive fields of the forelimb represented by the executant fibers. It was hoped that answers to these problems would indicate, at least in part, the integrative rôle of the known reflex effects.

Technique

The decapitate cat preparation was employed throughout the present study, the spinal cord being severed, during ether anaesthesia, by way of the dorsal atlanto-occipital membrane. Artificial respiration was instituted, and the anaesthetic discontinued. Individual nerves of the forelimb were prepared for "conditioning" stimulation, or, in some instances when a powerful afferent volley of mixed fiber origin would satisfy the requirements, components of the brachial plexus were employed for stimulation. Action, engendered in the hindlimb segments of the spinal cord by afferent volleys in forelimb nerves, was tested by the use of monosynaptic reflexes pertaining to individual hindlimb muscles. In order to secure such test reflexes the desired muscle nerves were freed and identified by the muscle contraction evoked when stimulated. Either the ventral roots or dorsal roots of the appropriate postthoracic segments were severed. According to the kind of roots severed, the monosynaptic test reflexes were obtained by stimulation of the individual muscle nerves while recording from a ventral root, or by stimulation of a dorsal root while recording from designated muscle nerves. In either circumstance the size of the recorded monosynaptic reflex discharge tested the average excitability of the particular motoneurons associated with the selected muscle nerve (8). Interchangeable use of the two methods

* A preliminary report concerning a number of the experiments herein described was presented at the Chicago meeting of the American Physiological Society, May, 1947 (12).

of recording monosynaptic reflex responses, impractical when conditioning and test reflex pathways are restricted to a single spinal region (cf. 8), was made possible in the present experiments by the fact that section of dorsal roots for test stimulation could lay no obstacle in the path of the afferent conditioning activity being studied.

With the conditioning and test systems in any given experiment determined, the procedure for obtaining each measurement of effect was to record a number of observations, with the test response alternately in isolation for control, and in combination with the conditioning action. The averages for control and conditioned values of the test response were extracted and the two compared, the conditioned value in each instance being expressed in per cent of the control value. In this fashion the effect of random fluctuation to some extent was overcome.

Preliminary Considerations

Concerning latency. In the illustrations that follow considerable variation in latency of long spinal action will be noted. This is due to wide variation in the lengths of the afferent paths, both conditioning and testing. Earlier measurements of latency (5) were made in the most favorable standardized conditions, with minimum afferent pathways established by the use uniformly of brachial plexus and dorsal root volleys. Under those conditions the latency of inhibition was 2.5–3 msec., while that of facilitation was 6–7 msec.

When allowance is made for the differing afferent conduction distances, it is clear that the central latencies of inhibition and excitation are similar in these and in the earlier experiments.

State of preparation. During the course of these experiments it was found that two functional states of the decapitate preparation could be recognized. The inhibitory reflex was invariably present, but the excitatory reflex proved more labile, being powerful in some preparations, absent or nearly so in others. Presumably the relatively great complexity of the excitatory pathway accounts for its susceptibility to state of preparation. As a matter of practical importance, variability between preparations makes some basis for comparison mandatory for each measurement of conditioning. For reasons that will become clear, the conditioning of flexor longus digitorum is the standard for tests of inhibition whereas the conditioning of gastrocnemius is the standard by which relative intensity of facilitation is gauged.

Activity in Motor Nuclei

In surveying the distribution to individual motor nuclei of long spinal reflex actions, afferent conditioning stimulation sufficient in strength to engage the whole range of myelinated fibers has been employed routinely so that comparisons of intensity might be valid.

Long spinal reflex discharge. It is known that long spinal reflex discharge is of variable intensity, not only from one preparation to another, but also in successive observations of an individual preparation (5). The frequent use

for recording purposes of the nerves to individual muscles has provided occasions for observing to which muscles the long spinal reflex discharge is directed. Of those examined it was the nerves of extensor muscles that received the discharges, a finding illustrated by Figure 1, in which records A-E show activity in the nerve of gastrocnemius, and records F-J show the absence of activity in the nerve to the combined ankle flexors.

Records, such as those of Figure 1, cannot reveal what, if any, change occurs in the flexor nuclei. Excitatory change necessarily would be of a mild nature not to result in discharge. Inhibition, the possibility of which is raised by considerations of reciprocal innervation, naturally would not be documented in the absence of a tonic discharge. Thus it is that recourse has been made to exploration by means of monosynaptic test reflexes of the individual motor nuclei.

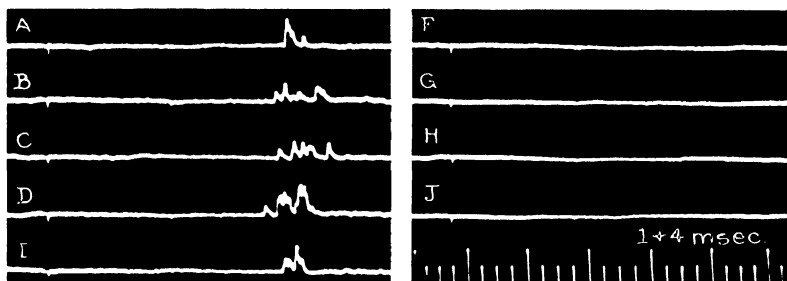


FIG. 1. Long spinal reflex discharge recorded in the nerve of gastrocnemius (A-E). Absence of discharge in nerves of ankle flexors (F-J).

Distribution among motor nuclei of long spinal reflex facilitation. Figure 2 illustrates an experiment in which was examined the influence, on the motor nuclei of gastrocnemius and tibialis anterior, of single shocks applied to the superficial radial nerve. Each point is located on the abscissae according to the interval, in msec., between conditioning and test stimuli. On the ordinates the points are located according to the conditioned size of the monosynaptic test reflexes expressed in per cent of control value. Gastrocnemius and tibialis anterior are antagonist members of the myotatic unit of the ankle (9, 10), deliberately chosen that their responses to long spinal reflex action might reveal a reciprocal relation if such were present. While it is apparent that motoneurons of the extensor gastrocnemius are strongly facilitated, those of its flexor antagonist seem not to be influenced.

Extensor longus digitorum, a flexor at the ankle, is the other proper antagonist of gastrocnemius. To be found in Figures 4 and 5 are curves that exemplify the response of extensor longus to long spinal reflex action. In neither instance is there a period of inhibition such as might be expected to match facilitation

of gastrocnemius. Actually the motoneurons of extensor longus are mildly facilitated. Figure 4 is taken from the same experiment as Figure 2.

The illustrated response of gastrocnemius motoneurons is quite generally characteristic of hindlimb extensor nuclei; those of tibialis anterior and extensor longus motoneurons are not characteristic of all flexor nuclei. Experiments, in examination of action in the nuclei of the knee flexors, show the degree of facilitation to be greater there than in the nuclei of the ankle flexors, but less than in the extensor nuclei. Figure 3 illustrates an experiment in which the long spinal reflex response of biceps femoris posterior motoneurons, to stimulation of the superficial radial nerve, is compared with that of gastrocnemius motoneurons.

As a means of summarizing the results of a number of observations, it is well to assign symbols to indicate the relative intensity of facilitation in the individual motor nuclei. If the facilitation of gastrocnemius motoneurons, illustrated in Figures 2 and 3, arbitrarily be designated $+++$, then $++$ would express that observed in the nucleus of biceps femoris posterior (Fig. 3), $+$ the slight facilitation detectable in the nucleus of extensor longus (Figs. 4 and 5), and 0 would denote no discernible conditioning. These symbols are employed in Table 1 to indicate the potentialities for long spinal reflex facilitation of the named motor nuclei.

Long spinal reflex inhibition. Rather extensive search by means of monosynaptic reflex tests has revealed the characteristic long spinal reflex inhibition as affecting, among the motor nuclei of hindlimb muscles, only that of flexor longus digitorum. Inhibition of flexor longus motoneurons, by afferent volleys engendered in the superficial radial nerve, is illustrated by Figures 4 and 5, the curves of which were obtained during the course of experiment with preparations in each of the two functional states. On comparison of Figures 4 and 5, it is seen that the onset and development of inhibition are similar in both instances. The subsequent course of conditioning reflects the state of the preparation. In the more excitable preparations inhibition is abrogated by intercurrent facilitation (Fig. 4, also Figs. 6 and 9). In the less excitable preparations inhibition wanes without obvious sign of interruption (Fig. 5, also Fig. 10).

Extensor longus has proved antagonist relation with flexor longus in a myotatic unit of digits (10). As a consequence, it is of interest to compare, as has been done in Figures 4 and 5, the long spinal reflex influence on motoneurons of extensor longus and flexor longus. As earlier noted, the motoneurons of extensor longus are mildly facilitated, but in temporal course, this mild facilitation is not related to the inhibition of flexor longus, as might be expected if reciprocal action were determined by the long spinal reflex mechanism itself. Motoneurons of extensor brevis, the other proper antagonist of flexor longus, react to afferent volleys in forelimb nerves as do those of extensor longus.

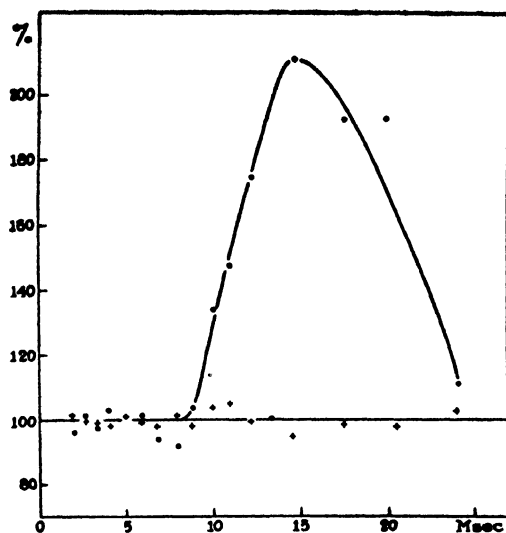


FIG. 2. Facilitation of gastrocnemius monosynaptic test reflex (dots), and absence of conditioning effect on test reflex of tibialis anterior (crosses), following stimulation by single shocks of the superficial radial nerve. This Fig. and Figs. 3, 4, 5, 6, 9, 10 and 11 are constructed in similar fashion. In each instance 100 per cent on the ordinates represents the unconditioned amplitude of the test monosynaptic reflex. Conditioned amplitude, expressed as per cent of control amplitude, is plotted as a function of time interval between conditioning and test stimuli. Points above the control value signify facilitation, points below signify inhibition.

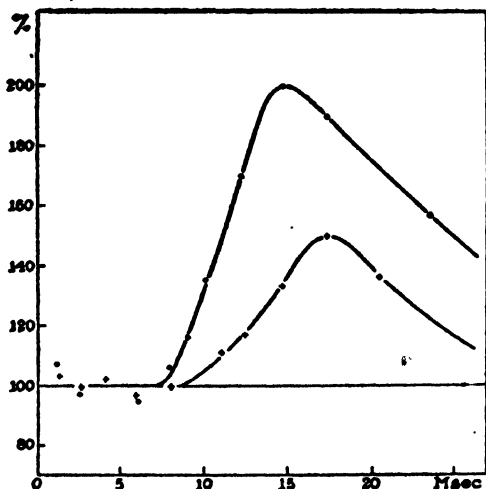


FIG. 3. Facilitation of the knee flexor biceps femoris posterior (crosses) compared with facilitation of gastrocnemius (dots) following single shock stimulation of superficial radial nerve.

TABLE I

Motor Nuclei of Hindlimb Muscles in Which Facilitation of Long Spinal Reflex Origin Has Been Demonstrated, and the Optimum Intensity of Facilitation Realised in Decapitate Preparations

Extensors*			Flexors*	
Of the hip:	semimembranosus	+++	biceps femoris posterior	++
Of the knee:	quadriceps	+++	semitendinosus	++
Of the ankle:	gastrocnemius	+++	tibialis anterior	0
Of the digits:	plantaris	+++	extensor longus digitorum	+ to 0
	flexor longus digitorum	+++	extensor longus digitorum	+ to 0
	flexor brevis digitorum	+		
To these may be added the ankle invertor, tibialis posterior,				+ to 0

* Functional rather than anatomical significance should be attached to these designations (cf. 16).

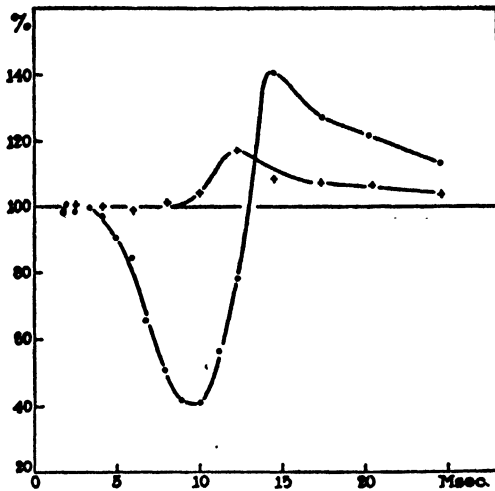


FIG. 4. Conditioning of flexor longus digitorum (dots) and extensor longus digitorum (crosses) by single shock stimulation of the superficial radial nerve. Preparation in the more excitable decapitate state. From the same experiment as Fig. 2. Compare with Fig. 5 illustrating similar experiment with a decapitate preparation in the less excitable state.

Plantaris and flexor brevis are closely linked together (10) and are allied with flexor longus for the performance of plantar flexion. Motoneurons of these muscles respond in long spinal reflex action as do all the others, excepting only those of flexor longus (Table I).

Finally, in suitable circumstances (for which see discussion in connection with Figs. 6, 7 and 8) it is possible to detect long spinal reflex inhibition in the absence of any other measurable effect in the motor nuclei of the hindlimb muscles. Long spinal reflex inhibition of flexor longus thus is the expression of a reflex mechanism distinct and separable from the others set into play by

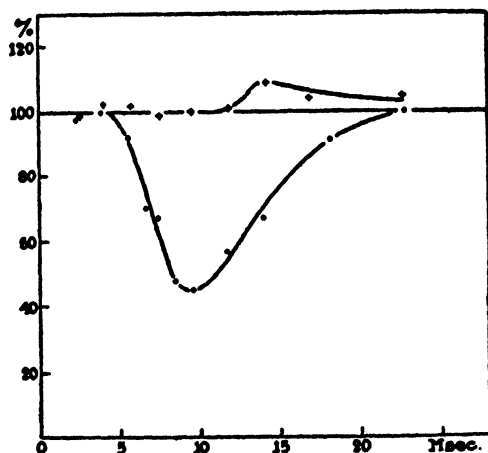


FIG. 5. Experiment similar to that of Fig. 4, but with decapitate preparation in less excitable state. Conditioning by single superficial radial nerve volleys of flexor longus digitorum (dots) and extensor longus digitorum (crosses).

forelimb nerve stimulation. Interpretation of this fact follows the further discussion of experimental observations.

Analysis of Afferent Channels

Two general methods exist for approximate identification of the afferent fibers that mediate spinal reflex reactions: (i) separate use of muscle and cutaneous nerves for afferent stimulation and (ii) relating intensity of response to the intensity of afferent stimulation (6).

Excitation and inhibition have different afferent thresholds. Illustration of this fact may be found in Figure 6. Contained therein are two curves that plot, as a function of time, the conditioning, by "strong" and "weak" afferent stimulation, of flexor longus motoneurons. Strong conditioning stimulation of the superficial radial nerve (crosses) initially inhibits and later facilitates the motoneurons of flexor longus. The preparation from which this experiment was obtained therefore was in the more excitable decapitate state' (cf. Fig. 4). The change in effect obtained by reduction in strength of the conditioning stimulation thus is significant; as seen in the curve represented by dots, it consists of a slight reduction of inhibitory intensity together with virtual

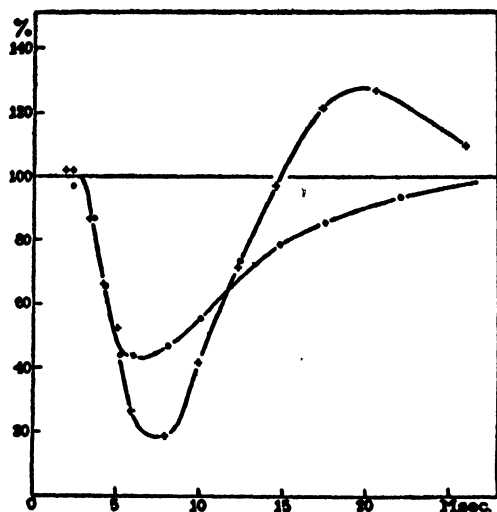


FIG. 6. Comparison of the conditioning effect on flexor longus digitorum of strong (crosses) and weak (dots) superficial radial nerve volleys.

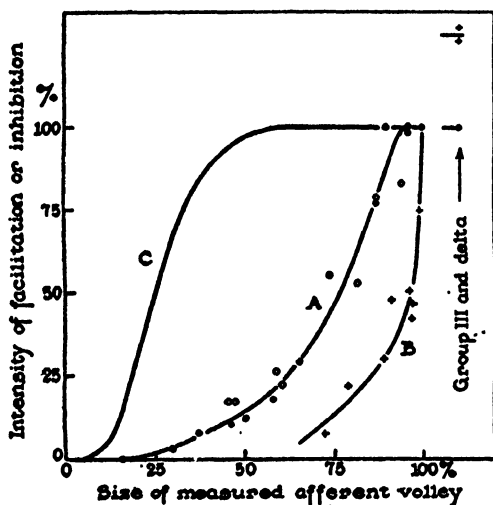


FIG. 7. Intensity of long spinal reflex inhibition and facilitation as a function of afferent conditioning volley size. A discussion of experimental conditions and definitions may be found in the text. Curve A: inhibition of flexor longus digitorum in decapitate preparations of the more excitable (dots) and less excitable (circles) states. Curve B: facilitation of gastrocnemius (crosses) in the more excitable decapitate preparation. At the right: added effects of including in the stimulated conditioning volley the contribution of Group III-delta fibers. Curve C: a curve relating monosynaptic reflex discharge to size of comparable afferent volley (cf. 6) to illustrate for purposes of comparison the shape of curve to be anticipated of action by Group I fibers.

disappearance of facilitation. Afferent threshold in the long spinal system therefore is lower for inhibition than for excitation, a relation that holds good when inhibition of flexor longus motoneurons is compared with facilitation of other motoneurons.

"Deep" nerves and cutaneous nerves are comparable afferent pathways. One means of segregating fibers for afferent stimulation is by the use separately of nerves destined to supply muscles and to supply skin. Nerves of the former category possess myelinated afferent fibers that, in size, are clustered into three peaks of numerical preponderance, known in descending order as Groups I, II and III (7, 11). There are two major fiber distribution peaks in cutaneous nerves (4, 13). Presently available information allows no distinction on the basis of reflex performance between Groups II and III of muscle nerves and the two groups of cutaneous fibers (7, 11). Group I is distinct from all the other fibers, whatever their peripheral origin, both by reason of reflex effect, and of the large size of its member fibers. It is of interest therefore to compare the long spinal reflex effects of afferent volleys arising in cutaneous and muscular nerves. In the forelimb the superficial and deep radial nerves* reasonably fulfill the requirement.

Qualitatively the long spinal reflex result in no way appears to differ by reason of employing the superficial or the deep radial nerve for conditioning. Indeed the result is similar when other nerves supplying the distal forelimb (the mixed median or ulnar nerves, or the medial cutaneous nerve) are substituted. Quantitatively however there is a marked difference for the deep nerve uniformly is much less effective as an afferent pathway than is the cutaneous nerve. The facts, (i) that deep and cutaneous nerves yield, through the long spinal system, similar effects, and (ii) that the deep nerve of the two kinds is much the less effective, suggest that, of the three groups of afferent fibers, Group I, of muscle origin, is not concerned with the long spinal reflexes. In the next section this inference is put to direct test.

Role of afferent fiber types in long spinal reflexes. One of the most fruitful methods for identifying the particular afferent fibers responsible for a given action is to relate the intensity of the action to the measured size of a causal dorsal root volley (6). It is not practical in the cervical region to employ a dorsal root volley. Instead, strands of the brachial plexus have been stimulated, the resulting afferent volleys being recorded at the root entry zone.

* The use of the deep radial nerve was dictated by the need of a large nerve for conditioning stimulation. "Deep" nerves may contain fibers distributed to other than muscle tissue, e.g., joints. It is known that the distribution of fibers according to diameter is in articular nerves similar to that of the Group II and Group III fibers in muscle nerves (3). To the extent that Group II and Group III afferent fibers are concerned in the long spinal effects of stimulating the deep radial nerve it is not valid to assume purely muscle origin for the efferent fibers.

Figure 7 presents the results of experiments in which the magnitude of long spinal effect is related to the size of afferent volleys of the sort described. Important for interpretation of Figure 7 is a clear understanding of the experimental conditions.

Curve A of Figure 7 represents the inhibitory conditioning of flexor longus motoneurons, curve B, the facilitatory conditioning of gastrocnemius motoneurons, measured by monosynaptic reflex tests, and resulting from brachial

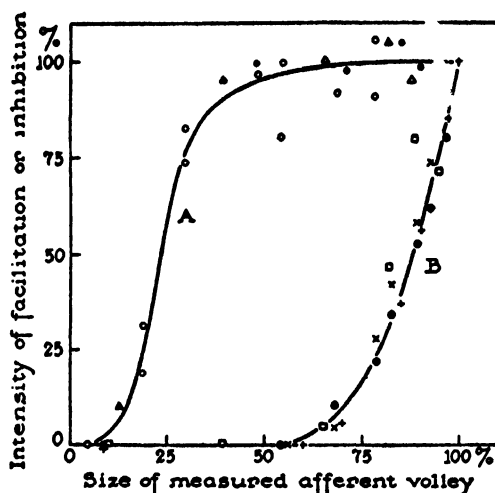


FIG. 8. Intensity of long spinal reflex inhibition and facilitation as a function of afferent conditioning volley size. In general similar to Fig. 7, but, by use of the superficial radial nerve for conditioning stimulation, Group I fibers are excluded from the measured afferent volleys. Effects of Group III-delta fibers also are excluded. Details of experimental conditions may be found in the text. Curve A: inhibition of flexor longus in four experiments the points from which are identified by distinguishing symbols. Curve B: facilitation of flexor longus (\square), biceps femoris posterior (\circ), gastrocnemius (\times) and ($+$) as observed in different experiments.

plexus volleys of various sizes. Fixed conditioning-test shock intervals were used. The recording of conditioning volleys of necessity was made at a point several centimeters distant from the stimulating cathode. It follows that volley dispersion would prevent Group III, or delta fibers from contributing to the measured potential of the afferent volleys, and would distort slightly the contribution of some of the larger fibers. Size of afferent volley, represented on the abscissae of Figure 7, therefore, is an approximate expression, in per cent of maximum, of the magnitude of an A fiber volley less its delta component. Since, among myelinated nerve fibers, spike amplitude varies with fiber diameter (4), the conditioning effect, as a function of the *number* of active

afferent fibers, is progressively condensed on the abscissa from left to right. An estimate of the participation of Group III-delta fibers can be made by noting whether or not added effect results on doubling the strength of a stimulus just sufficient to evoke the maximal measured afferent volley. Intensity of conditioning, inhibitory or excitatory, is expressed on the ordinates in per cent of the average maximal conditioning effect of the maximal measured afferent volleys.

Curve C of Figure 7 is a characteristic curve relating monosynaptic reflex discharge to size of afferent volley; it has the form to be expected of reactions depending exclusively upon the activity of Group I fibers, a notable feature being the fact that the reaction is complete by the time the afferent volley is half maximal (6).

Curve A of Figure 7 shows that inhibition of flexor longus motoneurons grows, with increasing intensity of stimulation, in a manner quite different from the growth of the known Group I reflex response of curve C, resembling rather the growth of response in multineuron reflex arcs (6, Fig. 2). Maximal inhibition, however, is realized by volleys short of maximal size. Facilitation of gastrocnemius motoneurons (curve B) occurs only by the use of volleys that are considerably stronger than threshold for inhibition of flexor longus motoneurons; it is less than half-maximum when provoked by volleys just sufficient for maximal inhibition of flexor longus motoneurons, and continues to increase with each increase in volley size. Further increase occurs on stimulation with shocks of delta strength.

Experiments of the type illustrated in Figure 7 lead to the following conclusions: (i) It is unlikely that Group I fibers are concerned with descending long spinal reflexes. (ii) Group III-delta fibers of the forelimb have, through the long spinal system, excitatory connection to hindlimb motoneurons, but are not concerned with the inhibitory reflex of flexor longus. (iii) Afferent fibers of long spinal reflex function, with the exception noted in (ii), reside in the fiber range represented by Group II of muscle nerves and the group of larger fibers in cutaneous nerves. Within this range of fibers there exists a degree of reflex differentiation documented for the first time in the distinctions between long spinal inhibition and excitation.

In view of the last of the preceding conclusions it is of interest to consider experiments like that of Figure 7, but differing with respect to the range of fibers contributing to the afferent volleys. For afferent stimulation in the experiments now to be considered the superficial radial nerve was employed and the distance between recording lead and stimulating cathode (ca. 2 cm.) was such that the potential contribution of the delta fibers did not add to the measured spike amplitude. At appropriate stimulation strengths the delta elevation could be seen to arise behind the measured afferent volley spike potential. Figure 8, presenting the results of these experiments, is constructed

in the same manner as was Figure 7, the outstanding difference being, by the use of a cutaneous nerve for conditioning, the exclusion of Group I fibers from contributing to the measured volleys. Volley size is expressed on the abscissa in per cent of maximum as defined above. As a consequence of eliminating Group I fibers, the curves are plotted on an "expanded" scale permitting more adequate differentiation of the inhibitory and excitatory reactions.

Curve A of Figure 8, relating intensity of long spinal reflex inhibition of flexor longus motoneurons to the size of afferent conditioning volley, shows that inhibition begins as the afferent volley exceeds approximately 10 per cent of maximum. With further increase in the afferent volley, inhibition increases rapidly to maximum at which time the afferent volley is still submaximal. The initial upward concavity of curve A, Figure 8, undoubtedly reflects the need for summation in the cervical cord relay before discharge of long propriospinal neurons is effected. The abrupt rise to maximum before the afferent volley is maximal indicates that only the largest cutaneous fibers contribute to the inhibitory arc.

Curve B of Figure 8 shows that the relation between afferent volley size and excitatory effect is quite different. Uniformly, facilitation of test reflexes, whether of flexor or extensor motoneurons, is not detectable until the afferent volley is approximately half-maximum. Facilitation, once established, increases in intensity with each increment in the afferent volley. Curve B has the form to be expected if only the smaller fibers of the range under observation establish connections through which excitation is projected to the final common path.

Receptive field of long spinal reflexes. Reflex responses in the forelimb are remarkable for the magnitude of their ipsilateral extensor component (1, 2). The extensor response classically is elicited by stimulation of the more distal nerves, or of the skin fields supplied by them; the contraction may be inhibited by stimulation of nerves more proximal. With the possibility that long spinal reflexes in similar fashion might exhibit distinguishing receptive fields, search was made for qualitative difference in long spinal reflex result depending on difference in the afferent fields of forelimb nerves. The forepaw was represented by the median nerve stimulated at the level of the wrist, the suprascapular cutaneous division (Reighard-Jennings) proved suitable to represent a proximal afferent field of the limb. Also utilized for afferent stimulation were the superficial radial nerve and the medial cutaneous nerve.

Between the excitatory reflexes of hindlimb flexors and extensors no significant difference in receptive field could be detected, intensity of effect in either case appearing to vary largely with magnitude of afferent inflow. By way of contrast the afferent field of the inhibitory reflex of flexor longus is highly restricted.

In Figure 9 are compared the actions on flexor longus motoneurons of afferent

volleys stimulated in the superficial radial nerve (dots) and in the suprascapular cutaneous nerve (crosses). The preparation was in the more excitable of the two described decapitate states. In each curve strong facilitation of flexor longus motoneurons is evident, but the inhibitory period, present if fibers from the distal forelimb are stimulated, is quite absent if the stimulated fibers supply only the proximal forelimb.

Figure 10 illustrates another experiment, the preparation for which was in the less excitable of the two decapitate states. The nerves for conditioning

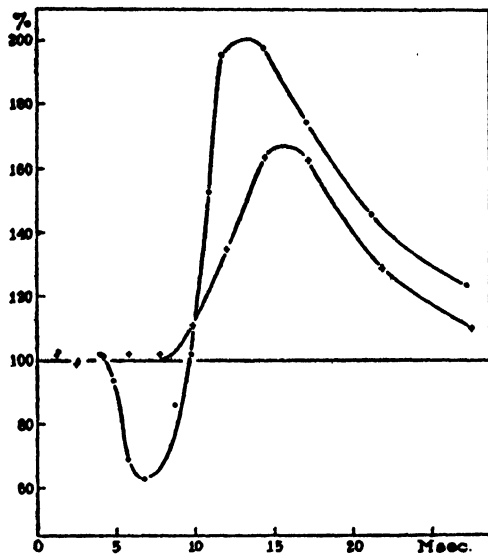


FIG. 9. Conditioning of flexor longus by superficial radial nerve volleys (dots) and by suprascapular cutaneous nerve volleys (crosses). Preparation in the more excitable decapitate state. Reflex inhibition occurs only with stimulation of the nerve to the distal forelimb.

stimulation were chosen to represent the extremes of the forelimb afferent field. In the upper part of the figure is seen a comparison of the effect on flexor longus motoneurons (dots) and on gastrocnemius motoneurons (crosses) of afferent volleys stimulated in the median nerve at the wrist. The lower part of the figure shows the effect on the same muscle pair, similarly identified by the distinguishing symbols, of afferent volleys in the suprascapular cutaneous curve. Comparison of the two parts of Figure 10 reveals that gastrocnemius motoneurons are similarly conditioned, and to about the same degree, by the distal and proximal forelimb afferent volleys. The conditioning result on flexor longus motoneurons, however, is converted from profound inhibition by the distal forelimb volleys into mild facilitation by the proximal forelimb volleys.

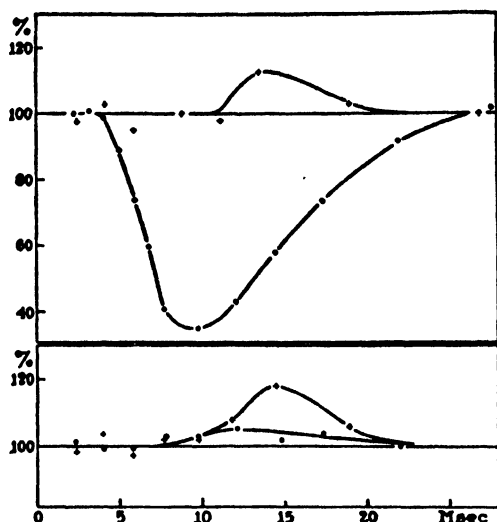


FIG. 10. Comparison of long spinal reflex conditioning of flexor longus (dots) and gastrocnemius (crosses). Preparation in the less excitable state. Above: conditioning volleys stimulated in median nerve at the wrist. Below: conditioning volleys stimulated in suprascapular cutaneous nerve. As stimulated these nerves represent extreme distal and proximal afferent fields in the forelimb.

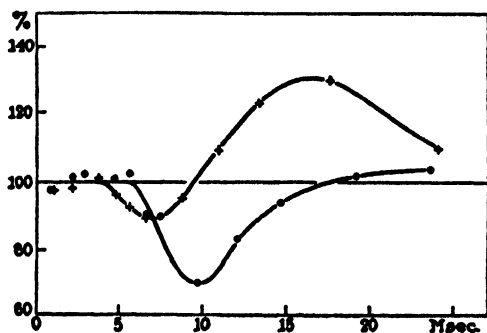


FIG. 11. Conditioning of flexor longus test reflex by stimulation of the median nerve at the wrist (dots) and by stimulation of the medial cutaneous nerve. The latency difference in onset of inhibition in the two curves is occasioned by the disparity in afferent conduction distance.

Further delimitation of the afferent field for long spinal reflex inhibition of flexor longus can be made by comparing, as is done in Figure 11, the results of stimulating the median nerve at the wrist (dots) and of stimulating the medial cutaneous nerve (crosses). As stimulated the nerves differ in the extent to

which the extreme distal forelimb is represented, the median nerve at the wrist being distributed exclusively to the paw, the medial cutaneous nerve supplying the forearm down to the region of the wrist. Of these nerves in several experiments the latter has proved uniformly the more potent source of excitation to hindlimb motor nuclei. Precisely the opposite relation holds with respect to inhibition of flexor longus, for in this action the median nerve, stimulated at the wrist, is the more potent.

Experiments such as those illustrated in Figures 9, 10, and 11 prove the receptive field for inhibition of flexor longus to be concentrated in the distal forelimb. Although it has not been possible unequivocally to limit the origin of the reflex to the forepaw, it is at least true that all of the nerves found on stimulation to provoke the inhibitory reflex contain afferent fibers from the region of the wrist or beyond. Afferent fibers that mediate the excitatory reflexes may arise anywhere in the forelimb.

COMMENT

Reflex function of long spinal reactions. By the use of monosynaptic test reflexes one may detect subtle excitability changes in motor nuclei. In the present experiments these have been evoked by gross stimulation of bared nerves. Concerning the evident conflict of excitations, a less sensitive indicator, motor discharge, in the first instance, may be more instructive for judging significance. It seems clear, from experiments of which Figure 1 is representative, that hindlimb extension would be in the acutely decapitate cat the dominant ipsilateral long spinal reflex. The flexor effects, in other language, would be concealed reflexes.

Thus, rather than suppose the excitatory actions on flexors and extensors to be parts of a single pattern, a simple and more plausible interpretation would hold that they represent an artificial admixture of two patterns. Evidence for this view is to be found in Table I. According to the results presented therein, long spinal excitation plays rather evenly over the extensor musculature of the hindlimb. Flexor excitation, by contrast, is markedly stronger at knee than at ankle or digits.

Without in any way identifying the excitatory actions with known long spinal reflexes, it is of interest to consider two of the latter because they illustrate opposite functional direction. Thus, stimulation of the forefoot pad, in the decerebrate preparation, causes the ipsilateral hindlimb, already extended in decerebrate rigidity, to extend even further, especially at the hip (14). On the other hand, the classical "hand-foot" reflex, obtained in chronic spinal preparations, is one of flexion at hip, knee and ankle. Particular note should be made of the differences in distribution or local sign between the classical reflexes and the actions described in this paper, for it is these differences that preclude identification.

It is clear that different reflexes may assume dominance in varied states of preparation—decerebrate, chronic or acute spinal. In the acute decapitate preparation employed for the present experiments, within the forelimb itself by inspection the flexor reflex has proved to be the overt response attending stimulation designed to evoke long spinal effects in the hindlimb. It is a not unreasonable assumption that the dominant reflexes evoked in forelimb and hindlimb by identical stimulation should be coupled reactions. Indeed, increase in the play of impulses upon extensor motoneurons of the hindlimb would be an appropriate concomitant of forelimb flexion, both for walking, and for maintained standing should the forelimb withdraw in response to nociceptive stimulation.

The inhibitory reflex of flexor longus must be regarded as a special response related to a particular rather than a generalized action of that muscle. In the general sense flexor longus acts in concert with the other physiological extensor muscles (16, 17). However, a unique function is not difficult to find. If, in a cat, the nerves to the muscles of the ankle and digits are isolated, severed, and the distal segments stimulated, it is found that flexor longus alone on contraction produces protrusion of the claws.* Since protrusion or unsheathing of the claws is a unique function of flexor longus, one may conclude that prevention of claw protrusion is the unique function of the inhibitory reflex of that muscle. In walking, operation of the inhibitory reflex would have the effect of removing claw protrusion from obligatory association with the extension phase of the step (16, 17).

Reciprocal innervation and long spinal reflexes. For responses evoked from the nervous system by nerve stimulation to be considered reciprocal it is not enough that reactions of opposite sign appear in antagonist muscles or in the motor nuclei representing them. In the clash of reflexes set into action, there is chance of an antagonist pair being influenced either similarly or contrarily. The latter circumstance might lead to a false conclusion of reciprocal action.

Included in the concept of reciprocal innervation is the notion of an identity of stimulus for excitation and stimulus for inhibition that approaches a demand for dichotomy of the executant structures (15). Reciprocal action in the monosynaptic paths of the myotatic unit is an example. Every known property of the myotatic unit (6, 8, 9, 10) is consonant with the idea that excitation and inhibition in the motor nuclei of its antagonist members arise by dichotomy of an action that contains initially the potentialities for both end results.

* This experimental finding is not in agreement with the conclusions of Straus and Sprague (19). They state that unsheathing of the claws in the "toe-spreading reflex" is produced by muscles innervated by the common peroneal nerve. However, they emphasize a passive rôle in claw protrusion of tension applied as a consequence of digital extension (*i.e.*, dorsiflexion or physiological flexion) to the tendons of flexor longus. It follows that active contraction of flexor longus would (and, as here shown, does) result in claw protrusion.

In long spinal responses the influences exerted on flexor and extensor longi are separable by threshold differences, and so do not qualify as reciprocal actions. None of the other described influences carries with it an expression of contrary action on antagonist motoneurons. One may conclude that the long spinal system as here studied in the acute decapitate preparation with the test arcs "opened" by root section does not possess some ingredient necessary for an expression of reciprocal innervation.

SUMMARY

Stimulation by single shocks of forelimb nerves evokes a variety of reflex effects in the ipsilateral hindlimb motor nuclei of the acutely decapitate preparation. The dominant excitor reflex is one of extension rather evenly distributed over the extensor musculature of the hindlimb.

A "concealed" type of excitor reflex concerns the hindlimb flexors. It is detectable in the decapitate preparation only by exploring with monosynaptic test reflexes the excitability of motor nuclei. This reflex is concentrated at the knee, rather than at ankle or digits.

Another reflex disclosed may be called the inhibitory reflex of flexor longus digitorum. The particular function of flexor longus is claw protrusion. Operation of the reflex in quadrupedal progression would have the effect of removing claw protrusion from obligatory association with the extensor phase of the reflex step.

Under the experimental conditions the foregoing reflex effects exhibit no sign of reciprocal action on antagonists.

Concerning afferent pathways the following conclusions may be drawn:

1. Deep and cutaneous nerves of the forelimb on stimulation yield the same reflex result.
2. Group I (myotatic reflex) afferent fibers do not have demonstrable long spinal reflex function.
3. A rather narrow band of the myelinated fiber spectrum, encompassing the lowest threshold cutaneous fibers (? alpha) and the lowest threshold Group II muscle afferent fibers is afferent for the inhibitory reflex of flexor longus digitorum.
4. Fibers afferent for the excitor reflexes lie among the higher threshold bands, including Group III-delta fibers.
5. Receptive field for the inhibitory reflex of flexor longus digitorum is limited to the extreme distal end of the forelimb.
6. Nerves from all parts of the forelimb contain fibers afferent for the excitor reflexes.

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LONG RANGE ENZYMATIC ACTION ON FILMS OF ANTIGEN

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The work reported here deals with the mode of action of two proteolytic enzymes, trypsin and pepsin, as well as that of the specific enzyme capable of depolymerizing the polysaccharide from Type III pneumococcus. This is a logical continuation of work previously published¹ on reactions between films of antigen and antibody molecules, where it was shown that specific forces between antigen and antibody seemed to extend considerably in space. In these experiments antigenic protein films were spread on water from which they were transferred onto polished metal slides. The slides were then coated with blankets of various inert materials and finally a drop of dilute solution of the corresponding immune serum was spread over each blanket. After the slide was washed, specific adsorption of antibody could be detected, the amount of which decreased regularly with the increase in thickness of the blanket. The probability that the antibody molecules reached the antigenic layers by diffusing through the blanket was for several reasons considered to be remote. The conclusion was therefore reached that the antibody molecules might actually be held on top of the slide by specific forces extending through the thickness of the blanket.

The analogy between immunological and enzymatic reactions naturally led to the question whether enzymes might not also exert their action through a blanket. In this case the interaction would be of such a nature as to bring about the breakdown of substrate molecules. Therefore, the film technique was extended to investigate possible long range enzymatic action.

When a slide covered with one or many monolayers of protein antigen is treated with a homologous immune serum, a specific adsorption of antibody occurs. However, no such adsorption takes place if the layers have been first treated for a few seconds with a pepsin or trypsin solution at the proper pH. Similarly, a monomolecular layer of the polysaccharide from Type III pneumococcus will adsorb a considerable thickness of homologous antibody but is no longer able to do so after being treated briefly with a solution of the enzyme which depolymerizes the polysaccharide. Thus the immunological reaction occurring between films of antigen and antibody molecules may be utilized as a very convenient and highly sensitive detector of enzymatic action.

The experiments devised to detect a long range enzymatic action were very

(1) A. Rothen, *J. Biol. Chem.*, **168**, 75 (1947).

simple. The antigenic layers—bovine albumin or polysaccharide from Type III pneumococcus—were deposited on a metal slide and coated with a screen of inert material, such as barium stearate or formvar (a formaldehyde polyvinyl polymer). A drop of the appropriate enzyme solution was then spread on the screen and allowed to remain for five to ten minutes. After the enzyme solution was washed off, the slides were treated directly with homologous antibody and the increase in thickness which followed was compared to that obtained on a screen of similar thickness when no enzyme treatment had been applied. (Advantage is thus taken of the long range interaction between antigenic films and antibody molecules to disclose a possible long range enzymatic action on the antigen films.) A far more satisfactory method, however, was to dissolve away the blanket after the enzyme treatment, and then to apply the antiserum directly onto the antigenic layers. It was found possible to remove formvar films with ethylene dichloride without impairing the immunological reactivity of either the protein antigen films or the adsorbed polysaccharide molecules underneath. The removal of the barium stearate screens was more difficult to carry out and was always accompanied by a partial loss of the immunological reactivity of the layers.

The experiments showed indeed, as reported in two preliminary notes,² that enzymatic action takes place in spite of an intervening blanket and that this action does not appear to result from actual diffusion of the enzyme molecules through the blanket. If our conclusions are correct it means that no intimate contact between substrate and enzyme molecules is necessary for the enzymatic action to proceed. This is of fundamental importance from a physical as well as from a biological point of view.

Experimental

The technique used in this work is essentially the same as that described in the preceding paper¹ dealing with immunological reactions. The thickness of transferred or adsorbed films was determined optically by measuring the change that occurs in the ellipticity of polarized light after reflection from a film coated slide. The instrument developed for this purpose is the ellipsometer, which is characterized by a half shadow end-point made possible by reference films deposited on the slides to serve as optical gage.³ Previous papers¹⁻³ should be consulted for the description of this instrument as well as for the experimental details concerning the preparation of the optical gages and their conditioning with uranyl acetate, the spreading of films and their transfer onto metal slides, the deposition of the blankets or screens, the treatment with antisera, the washing of the slides, and the optical measurement of the film thickness.

Enzymes.—Crystalline pepsin and crystalline trypsin in concentrations of 0.04% with respect to protein were used, unless otherwise stated. The samples of crystalline

(2) A. Rothen, *ibid.*, **163**, 345 (1946); **167**, 299 (1947).

(3) A. Rothen, *Rev. Sci. Instruments*, **16**, 26 (1945).

trypsin contained 60% magnesium sulfate. Pepsin was dissolved in 0.02 *M* hydrochloric acid and trypsin in a veronal buffer *pH* 7.5 (0.05 *M*). The solution containing the depolymerase for the polysaccharide was of unknown concentration but a range of dilutions from one to five was used.⁴ The medium was a phosphate buffer *pH* 7.2.

Antisera.—Antisera were diluted 0.1 with a phosphate buffer *pH* 7.2 containing 1% sodium chloride as described previously. Rabbit antbovine albumin sera and rabbit and horse antipneumococcus Type III sera were used.

Bovine Albumin Films—Antibovine Albumin Rabbit Sera. Pepsin Action.—Two to six unfolded monolayers of bovine albumin were transferred onto slides covered with an uranyl conditioned optical gage of one and three (or two and four) monolayers of barium stearate. A drop of pepsin solution deposited on the layers for one minute brought about a decrease in thickness from 8 to 30 Å. depending on the number of monolayers of bovine albumin. No increment in thickness occurred after subsequent treatment with homologous antiserum, which demonstrated that the remaining unfolded antigen films had been completely inactivated by the enzyme. A solution of 0.02 *M* hydrochloric acid alone could detach in one minute all layers transferred onto a slide except the first two. In this case, however, these remaining layers were not inactivated, as was indicated by an increase in thickness of 60 Å. after homologous antiserum treatment. When a blanket of one double layer of barium stearate about 50 Å. thick was deposited on two transferred double layers of bovine albumin, no change in thickness resulted from pepsin treatment and no adsorption of antibody occurred on subsequent antiserum treatment. If a drop of 0.002 *M* hydrochloric acid was substituted for the pepsin-hydrochloric acid solution, the antiserum treatment produced an increment of 40 Å. Similarly, slides with three double layers of bovine albumin coated with two double layers of barium stearate exhibited significant differences between those treated with hydrochloric acid alone and those treated with pepsin. A layer of antibody about 40 Å. thick could be adsorbed on the hydrochloric acid treated slide, but no change or even a considerable decrease in thickness occurred on the pepsin treated slides.

These experiments demonstrate that pepsin solutions are capable of destroying, through at least two double layers of barium stearate, the specific capacity of bovine albumin layers to react with antibody molecules. The fact, however, that the acid medium used for dissolving the pepsin is by itself capable of detaching some of the antigenic layers from the slides, made this enzyme unsuitable for a systematic research on account of the difficulty in differentiating the true enzymatic action from that of the medium.

Trypsin Action.—Trypsin proved an ideal enzyme to investigate, since a veronal buffer at neutral *pH*, in which the enzyme is most active, does not remove multilayers of protein from the metal slides except in one case which will be discussed later.

Conditioned slides covered with one, two or three double layers of bovine albumin were treated for three minutes with trypsin. A decrease in thickness of about 9 Å. occurred when there was one and a decrease of about 18 Å. when there was more than

(4) I am very much indebted to Dr. M. Kunitz for the samples of crystalline trypsin, and to Dr. O. T. Avery for the depolymerase solution.

one double layer of bovine albumin. No significant increase in thickness followed antiserum treatment. No change in thickness and no inactivation resulted from treating the slides with the buffer alone.

When one to three double layers of bovine albumin were coated with one double layer of barium stearate, no change in thickness followed trypsin treatment. There was an increase of 9 to 17 Å. when the blanket consisted of two or more double layers of barium stearate. Subsequent treatment with a homologous antiserum caused the removal of most of the stearate layers except when there was only one underlying double layer of bovine albumin, in which case no change or a very slight decrease of a few Å. units was observed.

The results showed that in spite of a blanket as thick as five double layers of barium stearate, the trypsin molecules were capable of undermining the architecture of two or three double layers of bovine albumin underneath. The anchorage of the blanket was weakened to such an extent that the barium stearate molecules were washed away by the antiserum treatment. It appears as if the foundations upon which the struc-

TABLE I

Ten Minute Trypsin Action through Blankets of Barium Stearate Multilayers on Three Conditioned Double Layers of Bovine Albumin (U Bov \uparrow), Deposited on a Gage of Barium Stearate

The figures in the table stand for the increase in Å. units observed after treatment with antiserum.

	Blanket, number of monolayers of barium stearate					
	0	1	2	4	6	10
Trypsin treatment.....	40*	70	30	15	18	10
No trypsin treatment.....	135	110	100	85	60	25

* 10 Å. in 20'.

ture of the layers of barium stearate were built had disintegrated under trypsin action. No such removal of the blanket took place after serum treatment if the slides were not submitted to trypsin action.

It was shown in a preceding article¹ that conditioning deposited layers of bovine albumin with uranyl acetate reduced the amount of antibody which could subsequently be adsorbed. In an analogous way, trypsin action on antigenic layers of bovine albumin is considerably reduced by conditioning. For example, inactivation of conditioned multilayers of bovine albumin does not occur following a two-minute trypsin treatment. The results obtained on inactivation by trypsin of three conditioned double layers of bovine albumin through intermediate blankets of barium stearate are summarized in Table I.

There was one conditioning treatment after each "round trip" (\uparrow) deposition but none after the last one. The system of layers can be represented by the symbol (U bov \uparrow)₃ where U stands for one uranyl conditioning. The trypsin solution was left for ten minutes on the slides. If we compare the figures of the two horizontal rows it appears that a significant difference in the thickness of the adsorbed layers of antibody, between the slides treated with trypsin and the slides not so treated, is still

noticeable with blankets up to three double layers of barium stearate ($\approx 150 \text{ \AA}$). These experiments indicate that the range of action of trypsin extends at least as far as the distances at which interaction between antigenic layers and antibody molecules can be demonstrated. By the very nature of the test, it cannot be said whether this action extends even farther. Uranyl conditioning between the deposited antigenic layers produced such a stabilizing influence that, in spite of the enzyme treatment, the blanket of barium stearate was never removed by the antiserum treatment. This stabilizing action of the uranyl ions may be twofold, first, in restricting the disintegrating effects set up in the bovine albumin layers by the enzyme, second, in holding together the fragments of the broken down molecules of the layers in such a way as to offer still a firm anchorage for the molecules of the blanket.

Obviously, in order to evaluate the maximum thickness of blankets through which trypsin molecules can act, the blanket should be removed after the enzyme treatment and prior to the deposition of the antiserum. It will be seen later that this can be accomplished easily with blankets of formvar. The removal of the blankets of barium stearate without impairing the optical gage of barium stearate underlying the antigenic layers proved a more delicate task. The problem was solved by using an optical gage of one and three, or three and five monolayers of octadecylamine instead of barium stearate. The slides were conditioned by uranyl acetate as usual. To remove the barium stearate blankets the slides were first treated with a citrate buffer pH 3.6 (0.057 *M*) for a minute or so to liberate the free stearic acid which was then leached off with ethylene dichloride or benzene. The optical gage of octadecylamine was unaffected by virtue of the insolubility of the salt of the amine in organic solvents.

Some experiments were made with blankets of octadecylamine which could be removed without affecting an optical gage of barium stearate layers. The blankets of amine were removed by treating the slides with a dilute solution of ammonia (0.01%) and then with benzene. Thus by taking advantage of the large difference in solubility in organic solvents between the salt of the acid and the free base (or between the free acid and the salt of the amine) it was possible to remove a blanket made of barium stearate without affecting an amine optical gage and vice versa.

The influence of blankets of octadecylamine on trypsin action have been summarized in Table II.

In all these experiments the blankets were dissolved before the application of the antiserum. It is shown in the table that the removal of the screen sufficiently disrupted the system of multilayers of bovine albumin so that even without enzyme treatment the thickness of the antibody layer which could subsequently be adsorbed was considerably reduced. The thickness of the antibody layer dropped from 185 to 73 \AA in the case of three double layers of antigen. The difference, however, between "buffer" and "trypsin" treated slides is sufficiently large to permit definite conclusions to be drawn. A screen of two double layers of octadecylamine completely protects one underlying double layer of bovine albumin. Five double layers of octadecylamine are necessary when there are two double layers of bovine albumin underneath. Finally, a blanket as thick as ten double layers was needed to protect three double layers of bovine albumin. It also appears that with two double layers of bovine albumin, whether the blanket had been one, two, or three double layers thick, the thickness of antibody adsorbed was about 17 \AA . This probably results from the

fact that the first antigenic layer directly attached to the gage is more resistant toward trypsin than the others. Indeed, without a blanket, multilayers were nearly completely inactivated in a few seconds; there remained, however, a small but definite power for adsorbing specific antibody, corresponding roughly to that of one single layer. Even after a three-minute trypsin treatment a specific increment of antibody of 10 Å. could still be observed. Complete inactivation occurred, however, in a few seconds if the slides coated with a conditioned gage were first covered with one single or one double layer of egg albumin before the deposition of the bovine albumin layers. This procedure thus ensured a more uniform sensitivity of the bovine al-

TABLE II

Six Minute Trypsin Action through Blankets of Octadecylamine Covering Multilayers of Bovine Albumin Deposited on Conditioned Barium Stearate Gages

The figures represent the increment in Å. units observed after treatment with the immune serum, following removal of the blanket. The subtitles "buf." and "try." indicate whether the slides were submitted to "buffer" or "trypsin" before the dissolution of the blanket. The duration of the "trypsin" or "buffer" treatment was six minutes. The letter R indicates that the blanket was detached by the trypsin treatment.

Blanket, number of double layers of octadecyl- amine	Number of bovine albumin double layers (↓↑)					
	1		2		3	
	Buf.	Try.	Buf.	Try.	Buf.	Try.
0	60	0	120	0	185	0
1	57	25		17		
2	38	38		17		
3	34	36		18		
4		37		48		R0
5			59	58		R0
6				54		
7			58			18
8				58	73	46
10					67	67
					or less	

bumin multilayers toward trypsin and it is for this reason that it has been used in nearly all the following experiments.

Some of the results obtained with screens of barium stearate have been summarized in Table III.

As in the experiments of Table II, the removal of the blanket produced partial inactivation (see Fig. 4 of ref. 1). The table shows that the greater the number of layers of bovine albumin the thicker the blanket needed for protection. It also appears that the mode of deposition of the antigenic layers is of importance. Inactivation could occur through blankets of greater thickness when the bovine albumin layers were deposited on the way up (↑↑)_n by successive emersions than when they were deposited by successive immersions and emersions (↓↑)_n. The depositions by emersion were accomplished by immersing the slides into the tray before the protein had

been spread. In the deposition by successive immersion and emersion the protein film was spread before immersing the slides. With the antigenic system ov $\uparrow\uparrow$ bov (\uparrow)₈, complete inactivation occurred through twenty double layers of barium stearate and twenty-five double layers were needed to ensure complete protection. This very fundamental difference in the behavior of "up" and "down-up" layers will appear even more strikingly in the results obtained with blankets of formvar described in the next section.

TABLE III

Six Minute Trypsin Action through Blankets of Barium Stearate Covering Multilayers of Bovine Albumin Deposited on Conditioned Gages of One and Three Layers of Octadecylamine

All slides after deposition of the blanket were treated for six minutes with a trypsin solution (active) or a solution of trypsin which had been brought to a boil for a few minutes (inactivated). After dissolving the blankets, the slides were treated with the antiserum. The figures stand for the increments observed in A units. The letter R indicates when the blanket was removed by trypsin treatment. "ov" and "bov" stand for ovalbumin and bovine albumin, respectively.

System of antigenic layers	Enzyme treatment	Blanket, number of double layers of barium stearate				
		1	2	3	4	5
ov \uparrow bov \uparrow	Active	25				
	Inactivated		45			
ov \uparrow bov(\uparrow) ₂	Active	15	26			
	Inactivated		46			
ov \uparrow bov(\uparrow) ₃	Active	0	2			
	Inactivated		57			
ov \uparrow bov(\uparrow) ₄	Active	0	0	0	0	0
ov \uparrow bov(\uparrow) ₅	Active	0	0	R	R	R
ov $\uparrow\uparrow$ bov \uparrow	Active	10				
ov $\uparrow\uparrow$ bov(\uparrow) ₂	Active	0	0	8		
ov $\uparrow\uparrow$ bov(\uparrow) ₃	Active	0	0	0		
ov $\uparrow\uparrow$ bov($\uparrow\uparrow$)	Active	0	18	36	42	45
	Inactivated		48			
ov $\uparrow\uparrow$ bov($\uparrow\uparrow$) ₂	Active	0	0	0		10
	Inactivated			86		
ov $\uparrow\uparrow$ bov($\uparrow\uparrow$) ₃	Active	0	0	0	0	0

Formvar Blankets.—Blankets of barium stearate or octadecylamine were not entirely satisfactory for two reasons, first the removal of the blankets produced a certain amount of inactivation, second the blankets were often removed by the enzyme treatment when there were three double layers of antigen underneath. Such disadvantages were not encountered with formvar blankets.

All the results obtained with screens of formvar have been summarized in Fig. 1. The abscissas give the thickness in ångström units of the formvar blankets present during the trypsin treatments and the ordinates, the amount of antibody adsorbed after removal of the blanket.

In other words, the phenomenon of cooperation between successive layers is very sensitive to small variations in experimental conditions.

Figure 1 summarizes experiments made with one, two and three double layers of bovine albumin deposited by the "down-up" process or the "up" process on slides covered with a conditioned gage of three and five layers of barium stearate plus one double layer of egg albumin. The abscissas show the thickness of the blanket of formvar upon which the trypsin solution was deposited, and the ordinates, the thickness of antibody specifically adsorbed after removal of the screen with ethylene dichloride. It is evident from the curves that the thickness of a formvar blanket needed for protection against trypsin action increases with the number of bovine albumin layers, in harmony with what was found with blankets of barium stearate and octadecylamine. A blanket of formvar about 180 Å. thick is necessary to protect three double layers of bovine albumin whereas for one double layer of bovine albumin 70 Å. only will suffice. It is to be noted that all curves rise very sharply ($\partial y/\partial x \rightarrow \infty$) at a critical thickness of blanket.

Time Factor.—In all these experiments, the trypsin solution was left on the blankets for ten minutes. The time factor was investigated with the system ov $\downarrow\uparrow$ bov ($\downarrow\uparrow$)₂. It was found that essentially the same curve was obtained whether the enzyme solution stayed for five minutes or fifteen minutes on the slides. The amount of specifically adsorbed antibody, however, was only 35 Å. instead of 80 to 85 Å. if the trypsin solution was left for four hours on a blanket 160 Å. thick deposited on one double layer of egg albumin plus one double layer of bovine albumin.

When the layers of bovine albumin were deposited on the way "up" curves 4, 6, 7, 8 and 10 of Fig. 1 were obtained. It is evident from comparing the results presented in Fig. 1, that the mode of deposition of the antigen layers has a tremendous effect on the thickness of the blanket needed for protection. A blanket of 100 Å. is sufficient to protect the antigenic pile ov $\downarrow\uparrow$ bov ($\downarrow\uparrow$)₂, but the protective thickness has to be increased to about 260 Å. when the antigenic pile has the structure ov $\downarrow\uparrow$ bov ($\uparrow\uparrow$)₂. The total thickness of the protein layers was the same in both cases.

A formvar blanket 600 Å. thick offered practically no protection at all for six layers of bovine albumin deposited on the way up and the impressive thickness of 1,000 Å. was necessary to keep the protein layers completely active. It was, therefore, of importance to determine whether or not a system of "up" layers without blankets was much more labile toward trypsin action than a system of "down-up" layers. If this were so, it could be argued that the difference in behavior of the two types of systems toward trypsin in the presence of a blanket was due to a small amount of trypsin which might have diffused through the blanket and would be capable of inactivating the "up" layers and not the "down-up" layers on account of difference in stability. The inactivation of the two types of multilayers without blanket, was therefore investigated as a function of the concentration of the trypsin solutions. The experiments were carried out at 4° for the following reasons. It was observed that at room temperature (20°) the veronal buffer was by itself capable of removing the equivalent of three to four layers of an "up" system of multilayers, but none at all if the layers had been built up by the "down-up" process. At 4°, however, the veronal buffer did not remove any of the layers of either system. It is of importance to note that a system of six "up" layers once coated with a formvar blanket does not lose any

thickness following a veronal buffer treatment of ten minutes at room temperature. In other words, unfolded bovine albumin molecules cannot diffuse through a formvar blanket. The results which have been summarized in Table IV show that a system of "up" layers is inactivated slightly faster than a system of "down-up" layers, but this small difference cannot account for the large difference in the thickness of formvar blankets needed for protection against trypsin action.

The case represented by curve 5 of Fig. 1 is particularly interesting. All the bovine albumin films described so far were formed on a water surface and then transferred onto the slides. Bovine albumin molecules, however, can be adsorbed directly by depositing a drop of solution on the metal slides. In this case whatever unfolding of the molecules takes place is not complete, since the average thickness of an adsorbed layer was consistently found to be between 17 and 18 Å. This thickness is equal to that of two monolayers of unfolded molecules. The thickness of the blanket needed for protection, however, was about 180 Å., twice the thickness necessary to protect two "up" layers.

TABLE IV

Inactivation of Multilayers of Bovine Albumin by Trypsin

Figures in columns 2 and 3 represent the thickness in Å. units of adsorbed antibody after treatment of the multilayers by trypsin solutions at 4° for ten minutes.

Concentration trypsin, mg. per cc.	ov ↓↑+ bov (↑) ₂	ov ↓↑ + bov (↓↑) ₂
0.4	0	0
.008	48	66
.004	80	95
.0008	160	160

Conditioning of the antigen layers with uranyl acetate greatly diminished the thickness of the blankets in formvar needed for protection, as is plainly shown in curves 9 and 11 of Fig. 1. Fifty ångströms of formvar offer no protection for three double layers of bovine albumin, but ensure nearly total protection for the system of layers (U bov ↓↑)₂.

Curve 10 shows that a blanket of formvar as thin as 20 Å. completely protected against inactivation one monolayer of bovine albumin deposited on the way "up."

Since the thickness of a blanket needed for protection increases with the number of layers of bovine albumin underneath, it was of interest to determine whether a thicker blanket was also needed if layers of egg albumin were substituted for some of the bovine albumin layers. As it will be seen later trypsin acted upon egg albumin layers just as easily as upon bovine layers in destroying their property of reacting with immune sera. The following systems of layers were deposited on conditioned gages of barium stearate: (ov (↓↑)₂ bov ↓↑), (ov (↓↑)₄ bov ↓↑) and (ov ↓↑ bov ↓↑ ov ↓↑). The curve of inactivation as a function of the thickness of the screen was very much the same for all three systems and intermediate between curves 1 and 3 of Fig. 1.

Polyvinyl Chloride Blankets.—A few experiments carried out with blankets of polyvinyl chloride indicated that the screening action was analogous to that offered by blankets of formvar.

Metallic Blankets.—Metallic blankets of gold were deposited by evaporation in high vacuum directly onto transferred films of bovine albumin. It was found, however, that a thin blanket of gold 20 Å. thick evaporated onto three double layers of bovine albumin prevented any specific adsorption of homologous antibody. (In the case of the polysaccharide from pneumococcus Type III, a specific adsorption of antibody still occurred through 50 Å. of gold.) It was impossible to remove the gold once it was evaporated on the protein layers. Thus slides coated with bovine albumin layers and a blanket of gold obviously could not be used to detect any trypsin action through the blanket because of the lack of means to determine whether or not inactivation had occurred. Of all the methods tested to detect trypsin action through metallic blankets the one most satisfactory was as follows. Gold films were deposited by evaporation *in vacuo* on clean microscope slides. Thin films of formvar were then deposited on top of the gold by dipping the slide into a solution of formvar in ethylene dichloride. When the formvar film was detached from the slide and floated on a water surface, the gold film adhered to the formvar, with the result that the gilded face of the formvar film was in contact with the water surface. Such gilded blankets were transferred from the water surface onto the antigenic films coating a metallic slide in such a manner that the gilded surface was on top. Trypsin solution was deposited on the gilded blanket for ten minutes. After washing off the enzyme solution the slides were treated with a strong jet of ethylene dichloride which dissolved the formvar and removed the gold at the same time. If the blanket was transferred with the gilded face in contact with the antigenic layers, the gold could not be removed by ethylene dichloride treatment. It was observed that a blanket consisting of a formvar film 130 to 200 Å. thick, plus a gold film 40 to 60 Å. thick, offered complete protection against trypsin to six "up" layers of bovine albumin. Partial protection was ensured if the gold film was ≈ 30 Å. thick, and with no gold, as it has been shown above, complete inactivation still occurred with films of formvar as thick as 600 Å.

Ovalbumin and Antiovalbumin Rabbit Sera

The inactivation of ovalbumin films by trypsin was not studied extensively, because the amount of rabbit antibody which can be specifically adsorbed is independent of the number of deposited ovalbumin layers (1) and, therefore, does not afford a sensitive indication of inactivation. Some of the results are presented in Table V, which shows that a blanket of 70 Å. of formvar offers ample protection for two as well as for four double layers of ovalbumin.

It should be noted, however, that if the ovalbumin layers were deposited on one double layer of bovine albumin complete inactivation occurred in spite of a screen of 64 Å. This would seem to indicate that a layer of ovalbumin anchored directly on the barium stearate gage is much more resistant to trypsin action than if it is anchored on a double layer of bovine albumin. As mentioned earlier, an analogous situation was encountered with films of bovine albumin.

Action of the Specific Enzyme Hydrolyzing the Polysaccharide from Type III Pneumococcus

The interaction between polysaccharide from Type III pneumococcus and homologous rabbit antibody through blankets of various kinds was described in the pre-

viously mentioned article.¹ In the present experiments horse as well as rabbit antisera were used, and interesting differences were brought to light.

The polysaccharide was adsorbed by placing a drop of solution on the slide, as described previously. The layer of polysaccharide, whether adsorbed on a conditioned gage of octadecylamine or on barium stearate, was about 5 Å. thick. When, however, the octadecylamine gage was not conditioned the adsorbed layer was 12 to 15 Å. thick, but the amount of antibody which could be specifically adsorbed was independent of the thickness of the layer of polysaccharide.

Horse Antipneumococcus Sera.—The thickness of the layer of horse antibody specifically adsorbed by the polysaccharide was about 70 Å. Reproducible results were difficult to obtain, values as large as 80 Å. and as low as 30 Å. were occasionally observed. The thickness was not dependent on the nature of the underlying gage, barium stearate or octadecylamine, nor did the coating of the gage with one double

TABLE V

Trypsin Action through Blankets of Formvar Coating Multilayers of Ovalbumin

The protein layers were deposited on conditioned gages of two and four layers of barium stearate. The blankets of formvar were dissolved in ethylene dichloride before treatment with the antiserum. The figures in the last column represent the thickness of the adsorbed antibody layer in Å. units.

Protein films	Thickness formvar blanket, Å.	Trypsin treatment, min.	Antiovalbumin rabbit serum treatment
ov($\downarrow\downarrow$) ₂	96	0	23
ov($\downarrow\downarrow$) ₂	71	5	18
ov($\downarrow\downarrow$) ₄	50	5	11
ov($\downarrow\downarrow$) ₄	74	5	18
bov $\downarrow\downarrow$ ov($\downarrow\downarrow$) ₂	64	5	0
bov $\downarrow\downarrow$ ov($\downarrow\downarrow$) ₄	60	5	0
bov $\downarrow\downarrow$ ov($\downarrow\downarrow$) ₄	51	0	20

layer of protein prior to the adsorption of the polysaccharide influence the results. These findings are in direct contrast to the events observed with rabbit sera where it was found that a layer of antibody about 300 Å. thick could be adsorbed if the polysaccharide was anchored on an amine gage whereas the increment was only 120 Å. if the polysaccharide was on a barium stearate gage. No increment was observed following treatment with an antiserum against Type I pneumococcus or upon treatment of an adsorbed layer of polysaccharide from Type I with a Type III antiserum.

When a drop of a solution of the depolymerase was left for ten minutes on a polysaccharide layer adsorbed on a barium stearate gage or on a barium stearate gage coated with one double layer of protein, complete inactivation of the polysaccharide occurred. The increment observed after treatment with an antiserum was from 0 to 10 Å. If, however, the polysaccharide was adsorbed on an amine gage or an amine gage coated with one double layer of protein, little or no inactivation resulted from the enzyme treatment, the thickness of the subsequently adsorbed layer of antibody being 30 to 60 Å. In other words, the anchorage of the polysaccharide was such that

the enzyme was ineffective. It could be argued that the amine by itself acted as an inhibitor for the enzyme. This assumption is disproved by the fact that if a blanket of octadecylamine is deposited on top of a polysaccharide adsorbed on a barium stearate gage, complete inactivation occurred through a blanket of at least one double layer of amine. It was also found that if one double layer of barium stearate was deposited on top of an amine gage prior to the adsorption of the polysaccharide, the enzyme was then capable of inactivating the polysaccharide just as well as if there had not been any amine layer underneath. Thus the direct anchorage of the polysaccharide to the amine is necessary to prevent the enzymatic action.

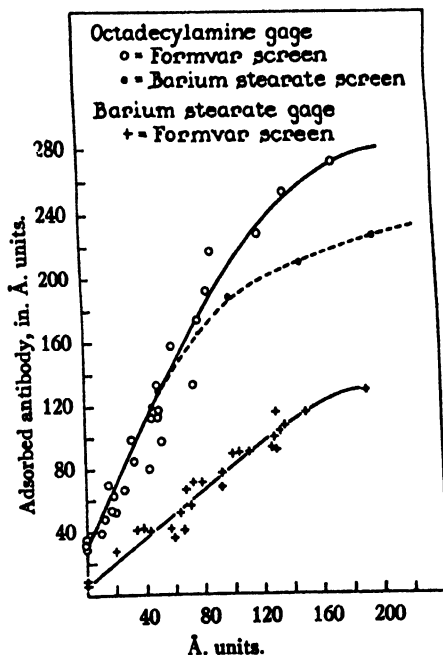


FIG. 2. Inactivation of polysaccharide from Type III pneumococcus through intervening blankets.

Rabbit Antipneumococcus Sera.—On account of the thick layers of rabbit antibody which could be adsorbed on a polysaccharide layer from Type III pneumococcus, this system was particularly suited for investigating the screening effect of blankets on the action of the depolymerase. The results have been summarized in Fig. 2, where the abscissa represents the thickness of the formvar or barium stearate blanket which was removed prior to the antiserum treatment. The curve shows that a blanket of about 180 Å. is necessary to protect the polysaccharide whether it was adsorbed on an amine or barium stearate gage. Also, a blanket of barium stearate has the same screening effect as that of an equivalent thickness of formvar. The same curve of inactivation was obtained whether the slides were treated for ten or twenty minutes

with the enzyme solution. With no blanket present the enzyme produced complete inactivation when the polysaccharide was adsorbed on a barium stearate gage, but on an octadecylamine base enough activity of the polysaccharide remained to produce an increment of 30 to 40 Å. of antibody, the same thickness found when horse serum was used. These experiments show that the enzyme is capable of altering the polysaccharide adsorbed on an amine layer to a much greater extent than the results with the horse serum indicated. The large increment of rabbit antibody adsorbed only a polysaccharide layer anchored to an octadecylamine gage results probably from a delicate adjustment between the structures of the antigen and the antibody. The small increment observed with the horse antiserum under similar conditions indicates that the horse antibody molecules do not respond to the whole antigenic pattern of the polysaccharide permitting the specific fixation of thick layers of antibody, but only to that part of the pattern responsible for short range action.

The enzyme is apparently capable of destroying the "fine" structure of the polysaccharide adsorbed on amine gage but cannot disrupt the whole of the pattern responsible for the specific reaction.

DISCUSSION

All the data presented indicate that pepsin and trypsin, as well as the enzyme depolymerizing the polysaccharide from Type III pneumococcus can act through various blankets made of formvar, polyvinyl chloride, barium stearate and octadecylamine. The question of fundamental importance to answer is: Do the enzyme molecules come in contact with the antigenic layers underneath, or do they act at a distance through the blanket by some mechanism yet unknown? A similar question was raised in the previous study on immunological reaction where it was concluded that the mesh of the fabric of blankets similar to those used in the present experiments did not appear large enough to permit the diffusion of the antibody molecules, and that all available evidence tended to indicate that long range action operated between antigenic layers located on one side of the blanket and antibody molecules adsorbed on the other side.

In the case of enzymatic action, one should keep in mind that a single enzyme molecule diffusing through a blanket might damage an extensive area of the antigenic film. It should also be remembered that the size of a trypsin molecule ($M \simeq 30,000$) is considerably smaller than that of a rabbit antibody molecule ($M \simeq 180,000$), and that nothing is known about the size of the enzyme depolymerizing the polysaccharide except that it is a non-dialyzable protein molecule.

However, the results obtained with trypsin acting on multilayers of bovine albumin layers through blankets of formvar offer the strongest kind of evidence against diffusion of the enzyme through the blanket. It was shown that the thickness of the blanket needed for protection against trypsin action increased with the number of underlying bovine albumin layers, and that the thickness

needed to protect three double layers of bovine albumin was nearly three times that needed for one double layer. It was shown also that the mode of deposition of the layers ($\downarrow\downarrow$)_n or ($\uparrow\uparrow$)_n was just as important as their total number. A much thicker screen was needed to protect a system of layers deposited upwards ($\uparrow\uparrow$)_n than a system of the same total thickness deposited by a round trip process ($\downarrow\downarrow$)_n. It is an amazing fact that a blanket 600 Å. thick offered no appreciable protection to six monolayers deposited upwards, a total thickness of only 48 Å. There is no reason to believe that the permeability of the blanket should depend on the number or mode of deposition of the antigenic layers underneath, especially when the fact is considered that the blanket can be made first on a clean glass slide, floated on water and transferred as one single unit onto the antigenic layers. If the enzyme molecules do actually diffuse through the blanket, they must then diffuse faster or slower depending on the mode of deposition and number of the antigenic layers underneath, a process which in itself would involve a long range action. The fact that the enzymatic action is to a certain extent independent of the time, the same degree of inactivation occurring after ten or twenty minutes in the case of the enzyme depolymerizing the polysaccharide, or after five or ten minutes with trypsin, speaks against a diffusion process. Curve 9 of Fig. 1 indicates that the amount of inactivation of uranyl conditioned layers of bovine albumin through blankets of formvar is the same after two or fifteen minutes. With no screen present, however, the thickness of the adsorbed layer of antibody for slides treated for two minutes with trypsin was more than twice the thickness obtained with slides treated for fifteen minutes. It may be that in the absence of a blanket, the trypsin molecules diffuse slowly through the disintegrating layers and are able to inactivate each layer in turn whereas no diffusion of enzyme molecules could take place through the blanket.

Slides covered with protein multilayers are hydrophilic; they become hydrophobic when the layers are coated with a formvar blanket and are still hydrophobic after trypsin treatment. One must assume, nevertheless, that water and buffer ions must diffuse through the blanket and that the range of action of trypsin molecules should depend on the diffusibility of the buffer ions. It is a fact that trypsin in a phosphate buffer acts at markedly longer distances than in a veronal buffer. There was no appreciable difference in the action of trypsin whether the formvar blankets were formed directly on top of the antigenic layers or whether they were transferred as one unit from a water surface. There was, however, a difference in the shielding action if the blankets were made in two steps, either by forming a blanket directly on the slide and then transferring a second blanket on top of it from a water surface, or by successively transferring two blankets from a water surface. When the antigenic layers consisted of six "up" layers, a screen of formvar 500 Å. thick made in two steps was adequate to protect the system from trypsin action. A con-

siderable amount of inactivation still occurred with a "two step" blanket 300 Å. thick. This difference in the behavior of the two types of blankets may be due to a diminished permeability to the buffer ions when the blankets have been made in two steps.

It has sometimes been questioned whether, during the treatment of the slides with enzyme solution or antisera, the different layers stay in their original position or whether these are sufficiently mobile to be displaced. Previously mentioned experiments showed that successively deposited layers remain in their order of deposition. It may be added that some of the experiments on the enzymatic action on the polysaccharide from Type III pneumococcus point to the same interpretation. The enzyme can completely inactivate the polysaccharide molecules adsorbed on a stearate layer deposited on an octadecylamine base. The inactivation is incomplete if the polysaccharide is adsorbed directly on an amine base. Consequently the polysaccharide stays on the barium stearate layer and does not diffuse downward toward the amine layer or vice versa the amine base does not migrate upwards. Also the fact that a blanket of amine, deposited on the polysaccharide adsorbed on a barium stearate base, does not prevent inactivation by the enzyme demonstrates that the polysaccharide molecules stay anchored on the stearate and are not displaced when the blanket of amine is deposited on top of them.

It was shown in the study on interaction between films of antigen and antibody molecules that the amount of specific adsorption in the presence of a blanket was practically independent of the nature of the blanket. In the case of the action of the enzyme on the polysaccharide from Type III pneumococcus, a barium stearate blanket has the same protective effect as that of a formvar blanket of equivalent thickness. A different condition prevails when trypsin acts on multilayers of bovine albumin. Inactivation occurs through a larger thickness of barium stearate than of formvar. The interaction between antigenic layers and trypsin is so strong that very often, as we have seen, the blanket of barium stearate is removed following enzyme or serum treatment. When the interaction is diminished by conditioning with uranyl acetate in between the deposition of the antigenic layers, the blankets of barium stearate are never removed and it was shown (Table II) that trypsin acts at least through six, possibly ten, monolayers of barium stearate blanketing three double layers of bovine albumin deposited by the round trip process. In contrast, a blanket of formvar 60 Å. thick entirely cuts off trypsin action on similarly treated layers of bovine albumin.

It has been suggested that the metal slides onto which the layers were deposited have such rough surfaces on a molecular scale, that the results observed might be artifacts due to the valley-mountain profile of the slide. It is known, however, from electron micrographs that an ordinary microscope glass slide has a remarkably smooth surface. Therefore, some glass slides were coated by

evaporation *in vacuo* with a film of chromium or gold thick enough to ensure metallic reflection. The same experiments were carried out using these slides and identical results were obtained.

The evidence just presented would indicate that the enzyme does not penetrate the blanket. Nevertheless, the effect of the enzyme does extend through the blanket and this fact should be considered in connection with theories of enzymatic action. The general mechanisms of enzymatic action postulated so far can be classified in two groups. One group assumes an intermediate complex between enzyme and substrate. The existence of such complexes has been demonstrated in the case of peroxidase and catalase by Chance.⁵ In the second group, the reaction proceeds from inactivation by collision and by taking a quantum of the energy liberated during the reaction.⁶

It would be very difficult to explain our results on the basis of either type of mechanism if contact between our substrate films and enzyme does not really occur. In the case of proteolytic enzymes, enzymatic activity does not seem to be located in a prosthetic group, but results from steric architecture of the whole molecule. The following experiments, which show that trypsin as well as pepsin requires the native configuration of its molecules to retain enzymatic activity, are in harmony with this view. One or two monolayers of unfolded trypsin deposited on a slide are incapable of inactivating subsequently transferred layers of bovine albumin, even if a drop of a buffer solution at pH 7.5 is deposited on the slide for ten minutes. Similar results are obtained if trypsin films are transferred on top of the antigenic films; no inactivation occurs. Unfolding of the enzyme molecule should not abolish its activity altogether if the origin of the enzymatic activity resided in a prosthetic group. Thus this activity must originate from an extensive portion of the active molecule.

One plausible explanation of the long range action at present would appear to be through some resonance phenomenon perhaps involving a characteristic frequency of the substrate and of the enzyme. Appropriate tuning could then result by correspondence in the frequency and polarization of the vibrations, and the vibrations set up in the substrate layers could break down certain bonds characterizing the antigenic pattern. This interpretation has already been proposed by Chaudhury,⁷ but is carried one step further. If such hypothetical resonators are extended, and as we have seen the evidence is in favor of an extended "active" part of the enzyme molecule, it is conceivable that resonance may occur at distances of an entirely different order of magnitude from those involved when small molecules or individual atoms are considered. Resonance might occur in spite of intermediate blankets, no immediate contact being

(5) B. Chance, *J. Biol. Chem.*, **151**, 553 (1943); *Acta Chem. Scandinavica*, **I**, 236 (1947).

(6) G. Medwedew, *Enzymologia*, **2**, 53 (1937).

(7) A. K. R. Chaudhury, *Curr. Sci.*, **14**, 261 (1945).

necessary between substrate and enzyme. It might also be conceived that, depending among other things on the frequency involved, certain vibrations would be better transmitted than others by the intermediate blankets. Some types of blankets may have a stronger specific adsorption than others for the particular frequencies involved. The experiments made with blankets of evaporated gold show that such blankets are extremely efficient in preventing enzymatic as well as immunological reactions.

Thus, long range enzymatic action through a resonance phenomenon could be an explanation of the observed facts. If, as the presented data seem to indicate, long range enzymatic action occurs between films of antigen, or adsorbed molecules of antigens, and enzyme molecules, it would seem also likely that the same mechanism could apply to a substrate in solution and should be considered in a discussion of any theory of enzymatic action.⁸

Also it is important to note that in these experiments one is not justified in considering the behavior of single molecules of the substrate independently. The effect of the number and mode of deposition of monolayers of bovine albumin on its range of action is already an indication that considerable interaction takes place between the layers and presumably between the molecules within one single layer. The phenomenon of long range action could be considered as due to the coöperation of a group of molecules. Coöperation phenomena may play a rôle in biological processes, the degree of coöperation possibly determining the distance at which an enzyme may act. Finally, the possibility of enzymatic action through a thin cell membrane offers a new vista on physiological events.

Most of the data presented in this work were obtained with the able assistance of Miss Marjorie Hanson. I am indebted to her for her help in the preparation of this article. My thanks go also to Dr. Lyman C. Craig who read with care the manuscript and offered valuable criticism.

SUMMARY

Multilayers of bovine albumin were submitted to the action of trypsin, and films of polysaccharide from Type III pneumococcus to that of a specific depolymerase. In both cases, following enzymatic action, the layers were

(8) It is interesting in this connection to mention an article by Vlasow, *J. Physics*, U. S. S. R., 9, 25 (1945). The author shows that when considering large polyatomic systems one cannot neglect weak forces of interaction at long distances and that "these interactions reveal new dynamic properties of polyatomic systems, putting the problem of the transition from 'micro' to 'macro' anew." When collective interaction is taken into account, then follows according to the author "the presence and spontaneous origin of eigen frequencies in polyatomic systems." It is worth mentioning that in 1939 Langmuir, *Proc. Phys. Soc.*, 51, 592 (1939), considered the possible importance of vibrations for the specificity of protein molecules.

altered to such a degree that they became incapable of specifically adsorbing homologous antibody. It was observed that blankets of barium stearate, of a plastic polymer (Formvar), and of polyvinyl chloride polymers deposited on the layers did not prevent enzymatic action from occurring when the enzyme solution was deposited on the blanket. The thickness of the blanket necessary to prevent any enzymatic action varied within a wide range depending on the number and mode of deposition of the underlying layers. It seemed unlikely that the enzyme molecules penetrated the blanket and the assumption was made that enzymatic action took place at a distance, the enzyme and substrate molecules being actually separated by an intervening blanket.

THE STIMULATORY ACTION OF CERTAIN FRACTIONS FROM BACTERIA AND YEAST ON THE FORMATION OF A BACTERIAL VIRUS

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Recent experiments have shown that the formation of bacterial viruses and the response of the host cells depend upon the composition of the culture medium. Wahl¹ has shown that some coli bacteriophages may be formed in synthetic medium without lysis of the host provided a certain concentration of thiamin and calcium is added to the medium. Raising the concentration of thiamin results in lysis of the host although no more virus is formed than without lysis. Experiments from this laboratory² have shown that *Staphylococcus muscae* phage can also be released in synthetic medium without lysis of the host. The addition of a substance present in veal infusion results in lysis of the host in the latter system. Fowler and Cohen,³ using a coli phage, have been able to increase the virus yield per cell by varying the composition of the medium. Maurer and Woolley⁴ have reported that the addition of apple pectin to synthetic medium permits the release of *E. coli* phage without cellular lysis.

It has now been found that a fraction from yeast or from the bacterial cell stimulates the formation of a bacterial virus. This fraction has a high concentration of ribonucleoprotein and has been referred to as the ribonucleoprotein fraction by other workers.⁵ The phage-stimulating property parallels the ribonucleoprotein fraction during purification. Splitting the ribonucleoprotein into free nucleic acid and protein causes complete loss of the phage-promoting activity. In view of these results, our working hypothesis is that the active substance is a ribonucleoprotein until experiments may prove otherwise.

A ribonucleoprotein fraction from yeast has recently been shown to stimulate the formation of adaptive enzymes in yeast.⁶ This nucleoprotein fraction has also been found to accelerate the formation of a bacterial virus. These results, together with a comparison of adaptive enzyme formation and bacterial virus formation, will be described in this paper.

¹ Wahl, R., *Ann. de l'Institut. Pasteur*, **72**, 73-80 (1946).

² Price, W. H., *J. Gen. Physiol.*, in press (1948).

³ Fowler, C., and Cohen, S. S., *J. Exp. Med.*, **87**, 259-274 (1948).

⁴ Maurer, F. D., and Woolley, D. W., *Proc. Soc. Exp. Biol. Med.*, **87**, 379-383 (1948).

⁵ Sevag, M. G., Lackman, D. B., and Smolens, J., *J. Biol. Chem.*, **124**, 425-436 (1938).

⁶ Reiner, J. M., and Spiegelman, S., *Fed. Proc.*, **7**, 98 (1948).

Experimental.—The *Staphylococcus muscae* phage system described previously was used in all the experiments.⁷ The cells and virus were grown as reported earlier.² Bacteria and phage were determined as in earlier experiments.⁷ The synthetic medium was the same as that described previously.³ One-step growth curves were carried out according to Delbrück and Luria.⁸

Most of the nucleoprotein fractions were prepared from bacterial cells first ground with powdered glass and then extracted with water. After centrifugation the supernatant fluid was adjusted to pH 4.0 with 10% acetic acid and 1 volume of alcohol added. The precipitate was collected, resuspended in water and adjusted to pH 6.3 with 0.5 M NaHCO₃. This solution was adjusted to pH 3.7 with 10% acetic acid. The precipitate was collected and the acid precipitation repeated. The yeast ribonucleoprotein fraction was prepared from fresh baker's yeast in a similar manner, except that the crude water extract was dialyzed against water for 10 hours before the alcohol fractionation. All operations were carried out at 5°.

Results.—The addition of the nucleoprotein fraction does not increase the per cent adsorption of the virus to the cell nor does it decrease the minimum latent period (Experimental Procedure in figure 1). It does increase the yield of virus per cell. As shown in figure 1, for the first 30 minutes the phage count, which represents the unadsorbed phage and the adsorbed phage, remains constant. This period is called the minimum latent period.⁸ At the end of 30 minutes, the phage titer goes up for 30 minutes as the virus particles are released by the infected cells. Due to the high dilution step used in the one-step growth curve, the phage titer no longer increases after the initially infected bacteria have released their phage, since there is no readsorption of the released virus particles to new cells. The average yield of viruses per cell may be calculated by the equation⁸ below.

$$\text{Average burst size} = \frac{\text{final virus} - \text{initially unadsorbed virus}}{\text{initial virus} - \text{initially unadsorbed virus}}$$

In the experiment shown in figure 1 the control tube formed an average of 7 viruses per cell and the tube containing the nucleoprotein fraction, 31 virus particles per cell. In the hundreds of tests that have been run, the nucleoprotein fraction from yeast or bacteria has increased the relative phage titer from 2 to 30 times over the control. The average stimulation is generally fourfold. It should be noticed that these tests are run with cells in the resting phase. Cells in the log phase form more phage than those in the resting phase and under these conditions the nucleoprotein fraction has only a slight effect, increasing the formation of phage not quite two times. It appears that this substance may be synthesized by rapidly growing cells. The experiment in

⁷ Price, W. H., *J. Gen. Physiol.*, **31**, 119-126 (1947).

⁸ Delbrück, M., and Luria, S. E., *Arch. Biochem.*, **1**, 111-141 (1942).

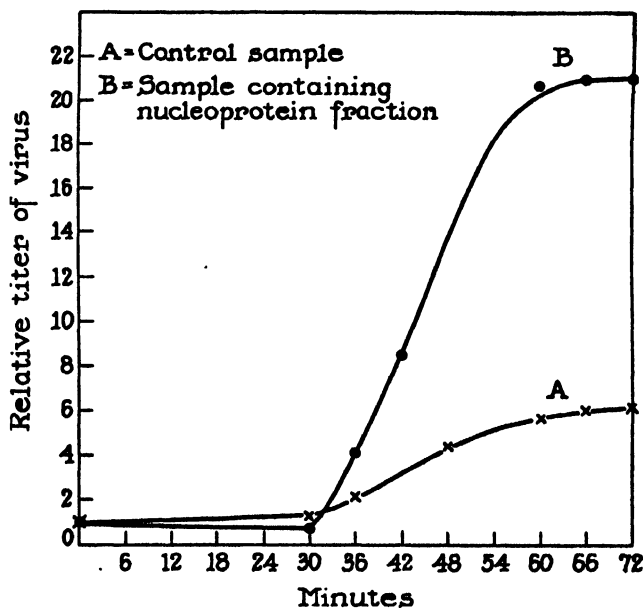


FIG. 1

The effect of the bacterial ribonucleoprotein fraction on the formation of bacterial viruses. The bacterial cells were washed off a 20-hour old veal infusion agar slant and prepared as described under Methods. Two tubes of synthetic medium containing 2.0 mg. of hydrolyzed casein were inoculated with 5.8×10^7 cells per ml. Tube A was the control and tube B received 0.1 ml. of the ribonucleoprotein fraction containing 0.7 γ of nitrogen per ml. After 1 hour of incubation both tubes contained 6.2×10^7 cells per ml. To each tube 0.1 ml. of a virus solution was then added to give a final titer of 4.8×10^7 particles per ml. The tubes were shaken 18 minutes and then diluted 1:2000 in their respective media. The control tube showed 43% of the virus adsorbed and the tube containing the nucleoprotein showed 40.7% of the virus adsorbed. Samples were taken from the diluted tubes for virus assay at various times.

TABLE 1

The Effect of Varying Concentrations of the Bacterial Nucleoprotein Fraction on the Formation of the Bacterial Virus

The same conditions were used as in figure 1. The values below represent the maximum titer at the end of a one-step growth curve. The initial titer was 2.1×10^4 plaque counts per ml.

Sample	γ N of Ribonucleoprotein fraction added per ml.	% Adsorption of virus to cell	Final plaque counts per ml.
1	—	40.2	1.1×10^5
2	0.22	42.1	2.1×10^5
3	0.44	38.6	3.1×10^5
4	0.88	41.3	5.3×10^5
5	1.6	39.1	5.6×10^5

table 1 shows the effect of varying concentrations of the nucleoprotein fraction on the formation of the virus.

The ultra-violet absorption curve of a highly purified fraction of the yeast ribonucleoprotein fraction shows a sharp maximum at 2600 A. U. and a minimum at 2450 A. The minimum at 2450 A is characteristic of nucleoproteins rather than free nucleic acid. The minimum of free nucleic acid is at 2300 A.⁹ Splitting the nucleoprotein into free nucleic acid and protein causes a complete loss of phage-promoting activity. In the present state of purity, the phage-stimulating activity of the ribonucleoprotein fraction is not destroyed on incubation with trypsin, chymotrypsin, pepsin, ribonuclease or desoxynuclease. It is, of course, not dialyzable. It is not precipitated by cold trichloroacetic or metaphosphoric acid, is very poorly precipitated by ammonium sulfate, and is maximally precipitated by acid at around pH 3.7. It is not spun down by centrifuging 1 hour at 24,000 r. p. m. at pH 6.8 in a Bauer-Pickels air-driven centrifuge. The best bacterial preparation contained 15.3% nitrogen and 2.6% phosphorus. The nucleic acid is all of the ribose type.

Ribonucleoprotein fractions prepared from calf thymus, pancreas and liver are inactive. The virus, which contains mostly if not all desoxynucleic acid, inactivated by mild heating or acid, has no phage-promoting activity under our conditions. The conditions used to inactivate the phage do not inactivate the ribonucleoprotein factor. Tobacco mosaic virus, which is a ribonucleoprotein, shows no phage stimulating activity in our system.

Several months ago in a short abstract Reiner and Spiegelman announced the isolation from yeast of a ribonucleoprotein fraction which stimulated the formation of adaptive enzymes in yeast.⁶ In view of the great similarity between their substance and the substance being studied in this laboratory, their adaptive enzyme fraction was tried in our phage system. Table 2 shows that it acted exactly like the fraction isolated by us and had approximately the same activity per mg. of nitrogen as our fraction. All three purified preparations sent to us by Dr. J. Reiner were active in our system. Our compound is not active in the adaptive enzyme system. This is not surprising since their substance must be isolated in the presence of galactose.

Discussion.—The experiments reported in this paper show that the addition of a fraction, from the host bacterium or from yeast, containing ribonucleoprotein increases the virus yield per cell of infected bacteria. The ribonucleoprotein fraction isolated from yeast by Reiner and Spiegelman⁶ which stimulates adaptive enzyme formation is very similar to the substance isolated in this laboratory. Indeed, it may replace it in stimulating phage formation.

It is difficult at the present time to understand the mechanism involved in the stimulation of the formation of a phage containing desoxynucleic acid by

⁹ Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, **30**, 101-148 (1946).

ribonucleoprotein fraction. A theory has been proposed by Spiegelman¹⁰ to account for the action of his ribonucleoprotein fraction in enzyme formation. We have no evidence for or against his theory at the present time.

It appears to us that two possibilities exist: (1) the factor acts as a precursor-like or nutrient substance or (2) it effects some reaction necessary in virus synthesis. As a working hypothesis, the latter theory is being tested. From the work of Caspersson¹¹ and Brachet¹² it appears that ribonucleic acid is somehow concerned in protein formation. This, together with the observation that the ribonucleoprotein fraction also increases enzyme formation, makes it a likely hypothesis that the fraction is somehow accelerating protein synthesis. Chemical determinations of cells grown in the presence of this fraction have so far shown no differences over control cells, and cells, even in the lag phase, do not multiply faster in the presence of the compound.

TABLE 2

The Effect of the Yeast Adaptive Enzyme Factor on the Formation of the Bacterial Virus

The same conditions were used as described in figure 1. The values below represent the maximum virus titer at the end of a one-step growth curve. The amounts of nitrogen used represent that concentration which gave 50% stimulation. The initial titer was 3.1×10^6 plaque counts per ml. The adsorption was approximately 40% in all samples.

Sample	Additions	Final plaque counts per ml.
1	—	1.1×10^6
2	Ribonucleoprotein fraction isolated in this laboratory (1.1 γ N per ml.)	2.7×10^6
3	Enzyme stimulatory factor (0.81 γ N per ml.)	3.1×10^6

Another interesting point brought out by these experiments is the similarity between adaptive enzyme formation and bacterial virus formation. This similarity was pointed out by Northrop¹³ and, although this view was not very popular, it should be reexamined in the light of new experimental evidence. An adaptive enzyme is formed by cells when grown in the presence of its substrate just as cells form phage when grown in the presence of phage. Before the production of either adaptive enzyme¹⁴ or virus,⁸ there is a lag period.

¹⁰ Spiegelman, S., *Cold Spring Harbor Symposia on Quantitative Biology* (Cold Spring Harbor, Long Island Biological Association), **11**, 256-277 (1946).

¹¹ Caspersson, T., *Symposia of the Society for Experimental Biology* (University Press, Cambridge), **1**, 127-151 (1947).

¹² Brachet, J., *Symposia of the Society for Experimental Biology* (University Press, Cambridge), **1**, 207-224 (1947).

¹³ Northrop, J. H., *J. Gen. Physiol.*, **23**, 59-79 (1939).

¹⁴ Stephenson, M., and Yudkin, J., *Biochem. J.*, **30**, 504-514 (1936).

Both enzyme¹⁴ and virus¹⁵ may be formed in non-viable cells. Cells which have the ability to multiply rapidly appear to form adaptive enzymes¹⁶ and viruses¹⁷ better than old cells. A ribonucleoprotein fraction from yeast stimulates both virus and adaptive enzyme formation. One of the great differences that has existed between adaptive enzymes and viruses has been the destructive effect of the virus on the host. Even this difference, however, is not a very sharp one, since it has been found that the pathological response of the host to the virus may be modified by varying the medium.^{1, 2, 4}

Competitive interactions in the cell exist in the formation of both adaptive enzymes and bacterial viruses. Thus Spiegelman¹⁸ has shown that the formation of an adaptive enzyme in yeast may cause decreases in the other enzyme systems of the cell. In the formation of bacterial viruses in the *E. coli* system, Cohen¹⁹ has presented evidence that the formation of the virus prevents the synthesis of cellular constituents. The very interesting observations of Monod²⁰ on the formation of adaptive enzymes in bacteria are important in this connection. On exposing a cell to two sugars simultaneously, only one adaptive enzyme was synthesized. A similar situation in phage formation has been found by Delbrück and Luria⁸ who infected a bacterium with two different viruses and found that only one multiplied. Thus in both adaptive enzyme formation and bacterial virus multiplication there may exist a mutual exclusion effect. Finally, there are the observations with a few exceptions, that in bacteria, viruses¹⁹ and adaptive enzymes²⁰ both need a source of external nitrogen for their formation.

¹⁵ Herriott, R. M., and Price, W. H., *J. Gen. Physiol.*, in press (1948).

¹⁶ Wooldridge, W. R., Knox, R., and Glass, V., *Biochem. J.*, **30**, 926-931 (1936).

¹⁷ Krueger, A. P., and Fong, J., *J. Gen. Physiol.*, **21**, 137-150 (1937).

¹⁸ Spiegelman, S., and Dunn, R., *Ibid.*, **31**, 153-173 (1947).

¹⁹ Cohen, S. S., *Cold Spring Harbor Symposia on Quantitative Biology* (Cold Spring Harbor, Long Island Biological Association), **12**, 35-49 (1947).

²⁰ Monod, J., *Recherches sur la croissance des Cultures bactériennes*, Actualités Scientifiques et Industrielles, no. 911, Hermann et Cie, Paris, 1942.

THE FORMATION OF BACTERIAL VIRUSES IN BACTERIA RENDERED NON-VIABLE BY MUSTARD GAS

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It has been found that *E. coli* B and *Staphylococcus muscae* after treatment with mustard gas [bis(β -chloroethyl)sulfide] can produce phage but apparently can no longer multiply. Most of the experiments were carried out on the *coli* system so that this will be discussed first and in more detail. Table I shows that at a mustard concentration of 0.8×10^{-8} M the phage formation is still 50 per cent or more of the untreated control but the number of organisms which can multiply in a veal-peptone infusion has decreased from 1×10^8 per ml. to about 1×10^5 . When the concentration of mustard gas was $1.25 - 2.0 \times 10^{-8}$ M there were no viable cells and the phage formed from these cells was still 5 to 20 per cent of the untreated control cells. In an occasional experiment after treatment with 1.25×10^{-8} molar mustard the undiluted suspensions showed a few (less than 10) viable cells.

Since the test for viability is the crux of the present problem, an explanation of the tests used is important. Both colony count and culture in liquid media were used in testing for viability. Colony counts were made 24 to 72 hours after plating out on veal-peptone-agar plates; the cells being (a) spread on the surface and (b) mixed with 42°C. veal-peptone-0.7 per cent agar, and then layered onto the plate (2). In the liquid culture serial tenfold dilutions of the organisms were made in rich media and shaken at 37°C. The different media tested were (1) veal infusion-peptone broth; (2) (1) + 0.3 per cent yeast extract; (3) (1) in which normal *E. coli* had been grown from 1×10^8 cells per ml. to 1×10^8 per ml. and then the cells removed by a Berkefeld N filter; (4) difco-brain-heart infusion; and (5) a "complete" media in which were most if not all the vitamins and growth-promoting agents in addition to amino acids, yeast extract, and peptone.¹ Dilutions of untreated cells calculated to contain only

¹ The "complete" medium kindly supplied by Dr. A. C. Braun and R. J. Mandle was made up of the following materials plus water to a liter of solution: 3 gm. of yeast extract, 5 gm. of peptone, 5 gm. of glucose, 0.025 μ g of biotin, 0.01 mg. folic acid, 5 mg. of *p*-aminobenzoic acid, 5 mg. of inositol, and 1 mg. each of thiamin, pantothenic acid, pyridoxine, niacin, riboflavin, and choline. Besides the above, 10 mg. of each of the following amino acids was added to the liter of solution: *dl*-lysine, *dl*-methionine, *dl*-threonine, *d*-arginine, valine, *dl*-histidine, *dl*-phenylalanine, *dl*-leucine, and tryptophane.

2 cells per tube became turbid in 24 hours in all the various liquid media. In a number of test runs using cells treated with varying amounts of mustard, all the various media showed the same number of viable cells for any given treatment. Therefore, only one medium, the veal infusion-peptone broth, was used in the majority of the experiments.

The viable cells present after treatment with 0.8×10^{-8} M mustard are not responsible for the phage formation in this suspension for the following reasons:

TABLE I

Tube No.....			I	II	III		IV	
H, final molar concentration.....			0 (Saline)	0 (1.25×10^{-8} M hydrolyzed mustard)	0.8×10^{-8} M		1.25×10^{-8} M	
Cells per ml.....			1×10^8	1×10^8	1×10^8		1×10^8	
Viability tests	Plates	Total cells spread.....	50	50	1×10^6	1×10^6	1×10^6	
		Colonies found.....	10-60	10-60	6	0	0	
	Liquid	Total cells in 5 ml. veal infusion.....	2	2	5×10^6	5×10^6	5×10^7	5×10^6
		Turbidity after 4 days.	+	+	+	-	-	-
Phage formation	Total cell concentration per ml.....		1×10^7	1×10^7	1×10^7	1×10^6	1×10^7	
	Viable cell concentration per ml.....		1×10^7	1×10^7	60	0	0	
	T ₁ phage concentration per ml. in suspension at start.....		2×10^7	2×10^7	2×10^7	2×10^6	2×10^7	
	Phage per ml. (final)...		4×10^{10}	2.1×10^9	2×10^9	1.8×10^8	4×10^8	
	Phage per cell (final)...		400	210	200	180	40	
	Phage per viable cell..		400	210	3.3×10^7	∞	∞	

* The difference between this value and the corresponding value of Tube No. II is probably accidental since other experiments showed no such difference.

1. There are too few of them. A single viable cell would have to produce about 10,000,000 phage particles to account for the observed result. Normal cells produce about 200 per cell.

2. Lysis and liberation of the phage occurs in an hour or two which does not allow time for much growth of the relatively few *viable* organisms.

3. The yield of phage is approximately independent of the inoculating phage concentration. This would not be expected if the viable cells were multiplying.

4. Diluting out the viable cells does not affect the phage yield per cell.

5. The capacity to form phage does not disappear at higher mustard concentrations where the number of viable cells is zero.

The addition of a few (0.1 and 1.0 per cent) normal cells to a broth suspension of mustard-treated *coli* did not revive any observable number of the latter for

the number of cells that grew up in 3 to 5 hours was the same as in the tubes containing no mustard-treated organisms.

E. coli B received from two different sources, Professor M. Delbrück and Professor A. D. Hershey, have given qualitatively similar results. Most of the

Materials and Procedures for Table I

Preparation of Suspension C.—*E. coli* B were grown from a small innoculum in veal infusion for 10 to 12 hours, then diluted in veal infusion down to $1-3 \times 10^7$ cells per ml. and grown up to 2×10^8 cells per ml. as judged by Klett colorimeter readings. These cells in the log phase of growth were then centrifuged and resuspended in same volume of 0.85 per cent saline - M/100 pH 8 phosphate buffer.

H.—0.01 ml. mustard gas (M.P. $14.2^\circ\text{C}.$) in 32 ml. saline in a 125 ml. glass-stoppered bottle and shaken hard for 15 seconds. The concentration of mustard was checked by the bromine titration (1).

Tube I.—2 ml. of C + 2 ml. of 0.85 per cent saline.

Tube II.—2 ml. of C left at $27^\circ\text{C}.$ for an hour followed by addition of 2 ml. of H which had stood an hour at $37^\circ\text{C}.$

Tube III.—2 ml. of C + 0.72 ml. saline and 1.28 ml. H.

Tube IV.—2 ml. of C + 2 ml. H.

After all the tubes had stood an hour at $27^\circ\text{C}.$, samples were diluted 1/10 in ice cold veal infusion.

Counts of Cells.—0.5 ml. of indicated cell concentrations in veal infusion was spread on a previously poured veal infusion-agar plate and then incubated at $37^\circ\text{C}.$ Colonies were counted after 24 and 72 hours. Seldom was there any change after 24 hours.

Viability in Liquid Media.—5 ml. of the dilutions in veal infusion containing the indicated cell concentration were shaken in 20 mm. \times 170 mm. tubes at $37^\circ\text{C}.$ for as long as 4 days. Turbid suspensions which exhibited a swirling sheen on shaking were considered positive. Clear tubes indicated no viable organisms.

Phage Formation.—To 5 ml. of the indicated concentration of organisms was added 0.1 ml. of filtered T_1 phage which was 50 times the desired final concentration. The tube was shaken at $37^\circ\text{C}.$ for 1 to 3 hours and then the phage concentration determined by plaque count. A phage control to which no cells were added was analyzed with each experiment.

Plaque counts were obtained after mixing a dilution of the phage with 3×10^7 per ml. log phase *E. coli* B in 0.7 per cent agar-veal infusion and layering 1 ml. of this mixture on a previously poured veal-agar plate (2). Plaques were counted after 12 to 24 hours' incubation at $37^\circ\text{C}.$

experiments have been performed with subcultures of the sample from Professor Delbrück. Besides the T_1 phage used for most of this work, T_{4r} and T_6 phage have also been formed from non-viable mustard-treated *coli*.

The phage-forming capacity of mustard-treated *coli* cells decreases with time. Even in veal broth the phage formation may be virtually zero in an hour or two at $37^\circ\text{C}.$ It is more stable at low temperatures.

In some instances non-viable mustard-treated *coli* can swell or elongate when placed in veal infusion medium. This was observed microscopically and also by turbidity measurements. Table II shows how the capacity for swelling and phage formation varied with increasing mustard gas concentrations. In experiments not shown in Table II the turbidity increase varied several-fold with different concentrations of mustard with no measurable effect on the phage formed per cell. After treatment with 1.5×10^{-3} M mustard there was no change in turbidity and the phage formed was 10 to 20 per cent of the yield with untreated cells. It appears from these various results that the phage formation does not parallel the capacity to swell.

Experimental Procedure for Table II

E. coli B were prepared as described for the experiments in Table I, centrifuged, resuspended in saline - M/100 phosphate buffer pH 8 to a cell concentration of 2×10^8 cells per ml. in one instance and 4×10^8 in a second. These were then mixed with saline and mustard gas, dissolved in saline so the final concentration was that shown in Table II. After an hour at 27°C., each suspension was divided into two equal portions and centrifuged at 7,000 R.P.M. for 10 minutes at 10°C. and resuspended in veal infusion. One portion was shaken at 37°C. and the turbidity determined every 15 minutes in a Klett-Summerson colorimeter employing a 66 filter. The other suspension was inoculated with 2 T_1 phage particles per cell and then shaken at 37°C. till the suspensions had cleared which was usually an hour.

TABLE II
Swelling of and Phage Formation of Mustard Gas-Treated E. coli B

Mustard gas concentration ($\times 10^{-3}$ M).....		0	0.5	1.0	1.5	0	0.8	1.15	Phage control
Swelling	Time of shaking in veal in fusion at 37°C.	Klett turbidity readings (66 filter)							
	0	20	17	17	15	42	36	34	
	1 hr.	65	27	20	16	137	45	34	
	3 hrs.					345	93	38	
	3.5 hrs.	365	84	24	14				
	10 hrs.					530	144	34	
T_1 phage formation from 1×10^8 cells per ml.									
Plaque count at 1×10^6 dilution.....		80	82	48	11	51	26	12	2
Per cent of zero mustard.....			100	60	14		50	24	4

Respiration studies following treatment with varying concentrations of mustard have been made parallel to estimations of phage formation. Little or no change in oxygen uptake was observed even when the phage formation had

been reduced to 10 per cent or less of normal cells by 2×10^{-3} molar mustard. CO_2 liberation, however, showed a measurable depression at 0.8×10^{-3} molar mustard and was about 50 per cent of normal following treatment with 1.3×10^{-3} molar. The phage formed in these two instances was 70 and 20 per cent respectively of the controls.

It was found that *Staphylococcus muscae* are not as susceptible to the action of mustard gas as the *coli* organism. However, as may be seen in Table III, approximately 98 per cent of the organisms were rendered non-viable for veal infusion media by treatment with 1×10^{-3} molar mustard. This treatment did not depress the phage formation to any appreciable extent. 2×10^{-3} M mustard apparently obliterated the phage-forming mechanism.

Materials and Procedures for Table III

The materials and methods used in the experiments on *Staphylococcus muscae* were the same as for *coli* except that Locke's solution was used in place of saline - M/100 phosphate for the medium in which the mustard treatment was performed. For the general methods of growth of the organism and its phage see reference 3.

TABLE III

Tube No.....		I	II	
Final molar concentration of mustard.....		None	1.0×10^{-3} M	
Cells per ml.....		1×10^8	1×10^8	
Viability test	Total cells in 5 ml. broth (4 tubes each).....	2	500	50
	Turbidity after shaken 4 days at 37°C.....	+	+	-
Phage de-termination	Cells per ml.....	1×10^8	1×10^8	10
	Initial phage count per ml.....	2×10^8	2×10^8	50
	Final phage count per ml.....	2.2×10^9	1.1×10^9	280
	Final phage count per cell.....	22	11	28

Over a dozen experiments have been performed on the *coli* system of which only one failed to show phage formation after mustard treatment. In eleven experiments on *Staphylococcus* four failed to show an increase in phage. Since the mustard-treated organisms are quite labile, this may have been responsible for the negative experiments.

DISCUSSION

A number of investigators (3) have presented evidence that phage can be formed by cells which are not actively dividing. Zinsser and Schoenbach (4)

reported a number of years ago that rickettsia multiplied in cells which were not viable but they failed to obtain growth of equine encephalomyelitis under similar circumstances. Anderson (5) concluded that after ultraviolet light treatment *E. coli* would not produce colonies but could form phage; however, no experiments have appeared to substantiate this conclusion.

There can be no doubt from the present results that mustard gas has altered the cells. It is not possible to prove beyond any doubt that the cells are strictly non-viable or dead and cannot be revived for this would require an infinite number of tests, but the media and nutrients used have been varied enough to indicate that they do not multiply when placed under conditions very favorable for phage and cell multiplication. It follows from our results that the capacity for cell division is not necessary for phage formation.

The nature of or the site of the mustard reaction in the cell is not known but one of us has found (6) that bacteria exhibit the same order of sensitivity to mustard as such nucleic acid and nucleoproteins as the pneumococcus-transforming principle and animal, plant, and bacterial viruses. Most enzymes are much less sensitive.

SUMMARY

E. coli B and *Staphylococcus muscae* rendered non-viable by aqueous solutions of mustard gas at pH 7.5 to 8 can still produce phage.

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ISOLATION OF CRYSTALLINE RICIN*

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1. INTRODUCTION

A crystalline material possessing powerful toxicity has been isolated from crude extracts of castor bean meal. The crystalline material is a protein of the globulin type.¹ It is soluble in acid or alkaline solution and is least soluble in the range of pH 5.0 to 8.0. The ultraviolet light absorption spectrum of the crystalline protein is similar to that of a typical protein. The toxicity of the isolated crystals is higher than that of the mother liquor freed of the crystals. On repeated recrystallization the toxicity of the mother liquor approaches that of the crystals. Ultracentrifuge and electrophoresis measurements of a sample of three times recrystallized material showed that the material is fairly homogeneous. Solubility measurements, however, indicate that the crystalline protein even after several recrystallizations apparently consists of a solid solution of more than one component. The method of separation of the crystalline protein into its possible components is still unavailable.

2. Method of Isolation of Crystalline Ricin

The crystallization of ricin from crude extracts of castor bean meal proceeds best in the presence of sodium sulfate or ammonium sulfate.

(a) *Crystallization in the Presence of Sodium Sulfate*.—A solution of crude Na_2SO_4 -ricin² in water yields crystals of toxic protein when stored for several weeks at about 5°C. The details are as follows:—

10 gm. of dry powder of Na_2SO_4 -ricin is suspended in 30 to 40 ml. water. It is best to add the powder slowly to the measured amount of water so as to allow the powder to become wet gradually. The mixture is stirred until uniformly dispersed. It is then filtered clear on fluted paper. The filtration, if slow, is allowed to proceed overnight in the cold room at about 5°C. The solu-

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The experiments referred to were first reported July 15, 1944.

¹ The isolation of a non-toxic crystalline globulin from castor bean has been reported by Ritthausen in 1881 (1) and by Osborne in 1892 (2).

² This preparation is an aqueous extract of castor bean meal, precipitated by saturation with sodium sulfate (3). The precipitate is filtered off and dried.

tion is adjusted with 1 M sodium hydroxide or hydrochloric acid to pH 6.8^a and stored at 5°C. A slight precipitate of rosettes of very fine needles generally appears within 10 days (or longer). The bulk of the precipitate increases gradually until it reaches a maximum in 6 to 8 weeks. Occasional stirring accelerates the rate of formation of crystals, which is also greatly increased on inoculation with a large amount of crystals. The crystals are filtered or centrifuged. The yield is about 0.5 gm.

Recrystallization.—The crystalline precipitate is suspended in a volume of water equal to about one-fourth of the volume of water used for the first crystallization. Enough 1 M hydrochloric acid is added slowly with stirring until the crystals dissolve. The solution is filtered clear on fluted paper and the paper is washed with a small amount of 0.01 M hydrochloric acid. The filtrate and washings are titrated to pH 6.8 with 1 M sodium hydroxide, but the addition of sodium hydroxide is interrupted at the first appearance of turbidity even if pH 6.8 is not reached. The solution is stored at 5°C. A heavy crop of crystals generally is formed within 24 hours and the crystallization is complete in 2 or 3 days.

(b) *Crystallization in the Presence of Ammonium Sulfate.*—10 gm. dry Na_2SO_4 -ricin powder is stirred up with 30 ml. of water. The mixture is filtered on fluted paper; the residue on the paper is washed with about 10 ml. of water. Enough solid ammonium sulfate is added to the combined filtrate and washings so as to bring the solution to 0.8 saturation (5.6 gm. per 10 ml.). The precipitate formed is filtered with suction. The filter cake is weighed and then dissolved in water in proportion of 1.3 ml. of water to 1 gm. of filter cake. Saturated ammonium sulfate is added slowly until a slight turbidity appears which is removed by filtration through folded paper. The filtrate is titrated to pH 6.8 with 1 M sodium hydroxide. The solution is left for 2 to 3 days at 5°C. A heavy precipitate gradually forms. The suspension is centrifuged at a temperature not higher than 10°C. The residue is dissolved in about 3 ml. of water and stored at 5°C. A heavy amorphous precipitate forms in a few hours. The amorphous precipitate gradually changes into fine needles. The suspension is filtered or centrifuged after 2 or 3 weeks.

The method for recrystallization is the same as described in section (a).

The mother liquors from the various crystallizations generally yield more crystalline protein when brought to 0.8 saturation with solid ammonium sulfate. The precipitate formed is dissolved in about an equal weight of water and stored at 5°C. A heavy crop of crystals is formed within 2 or 3 weeks.

3. Form of Crystals

The material generally crystallizes in the form of rosettes of fine needles (Fig. 1 a). Large prismoidal crystals appear if recrystallization takes place slowly from dilute solution (Fig. 1 b).

^a The pH is checked by the drop method on a test plate.

4. Toxicity Tests

The toxicity of the first crystals was tested by Professor A. H. Corwin. 1 mg. of the crystalline protein was found to have a toxicity equivalent to 2 mg.

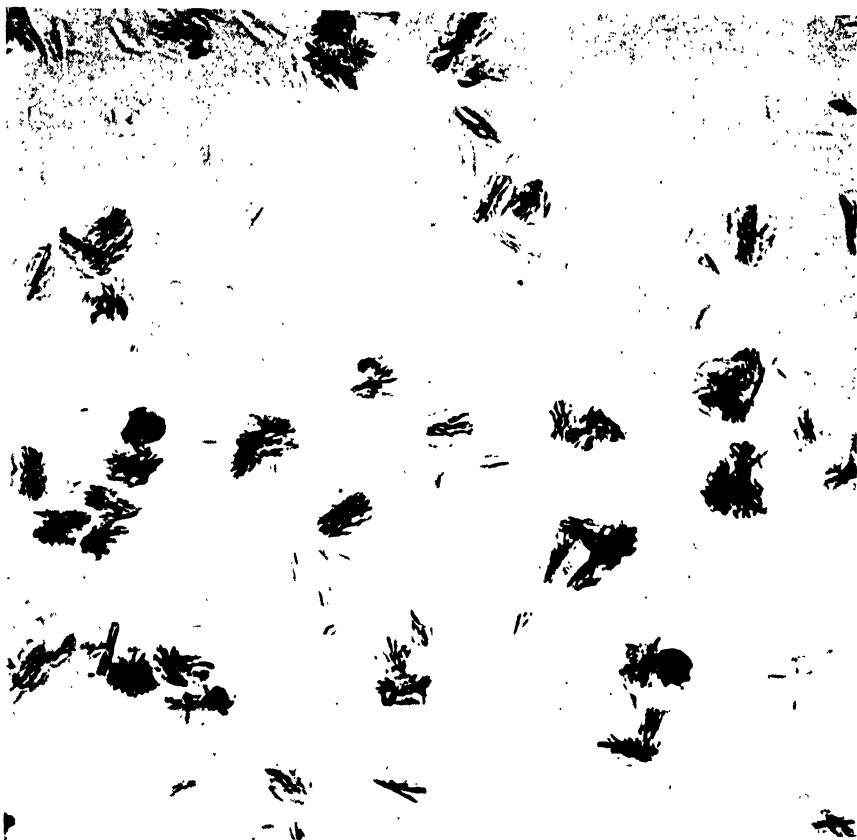


FIG. 1 *a*

FIGS. 1 *a* and 1 *b*. Crystals of ricin.

of protein of the crude Na_2SO_4 -ricin, whereas the toxicity of 1 mg. of protein in the mother liquor of the same crystals was equivalent to only 1.3 mg. protein of Na_2SO_4 -ricin. This indicated a sharp fractionation in favor of the crystals and is evidence that the toxicity is really a property of the crystalline protein.

The toxicity of three times recrystallized ricin protein was measured in Dr. R. Keith Cannan's laboratory. The toxicity of the crystals was 680 T.U. per mg. protein while the toxicity of the mother liquor was 520 T.U. per mg. protein.

The specific toxicity of five times recrystallized protein and of the mother liquor was determined in Dr. R. Keith Cannan's laboratory. No significant difference in the specific toxicity of the crystals and the mother liquor was found. This result shows that the present method of recrystallization no longer



FIG. 1 b

changes the composition of the crystalline protein sufficiently to be detected by differences in toxicity.

5. Some of the Protein Properties of Crystalline Ricin⁴

Crystalline ricin appears to be a protein of the globulin type, with an isoelectric point reported (3) to be at pH 5.4 to 5.5. It is least soluble in the range of pH 5.0 to 8.0. It is precipitated in 0.15 M trichloroacetic acid. Its ultra-

⁴ Additional information on the properties of crystalline ricin is to be found in the publication of Kabat, Heidelberger, and Bezer (3).

violet absorption spectrum (Fig. 2) resembles that of other proteins with a maximum absorption at a wave length at 279 $m\mu$ and a minimum at 250 $m\mu$.

6. Purity of Three Times Crystallized Ricin

(a) *Ultracentrifuge Method* (Dr. M. A. Lauffer).—Solution used: 1.5 per cent solution of three times crystallized ricin in 0.2 M sodium chloride made up in 0.05 M acetic acid; final pH about 3.5. The solution was filtered clear on No.

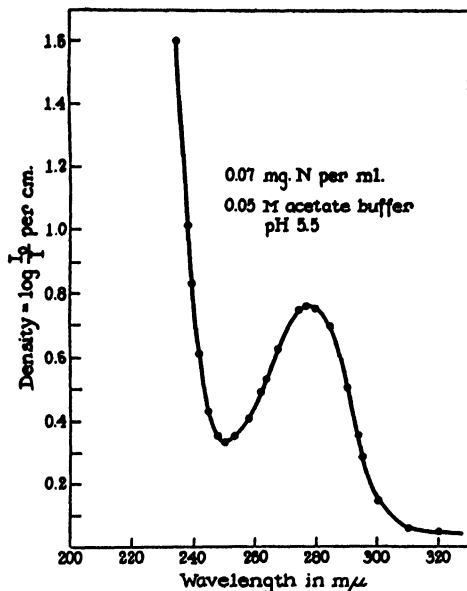


FIG. 2. Ultraviolet absorption spectrum of crystalline ricin.

42 Whatman paper. The data were recorded by the Svenson schlieren method at 10 minute intervals. Only one moving boundary was observed throughout the 2 hours' centrifugation and the symmetry of the schlieren curves indicated fair homogeneity of the material.

Sedimentation constant corrected to water at 20°C. = 3.9×10^{-13} cm./sec./unit field. Molecular weight = 36,000 (assuming the particles to be spheres with a specific volume of 0.73).

(b) *Electrophoresis* (Dr. M. A. Lauffer).—Solution used: 0.63 per cent solution of three times crystallized ricin in 0.02 M sodium chloride made up in 0.05 M acetic acid. The same electrolyte solution was used to fill the upper compartments of the Tiselius apparatus. Current passed at the rate of 10 milliamperes for 1.5 hours. Boundary recorded at the end of the experiment by the Longworth schlieren scanning method. The electrophoretic pattern indicated the presence of only one moving component.

(c) *Solubility Test.*—Measurements were made of the solubility in 0.05 M acetate buffer pH 5.5 of four times recrystallized ricin in the presence of increasing amounts of crystals of ricin in suspension.

Experimental Procedure.—5 gm. of three times recrystallized filter cake were dissolved in 15 ml. 0.1 M acetic acid and filtered clear. The solution was brought to pH 5.5 by means of 15 ml. 0.1 M sodium hydroxide and left for several days at 5°C. for crystallization. The suspension of crystals was filtered on Whatman's No. 42 paper at about 20°C. The crystals were then washed twice by resuspending in 30 ml. 0.05 M acetate buffer pH 5.5 and refiltering on No. 42 paper.

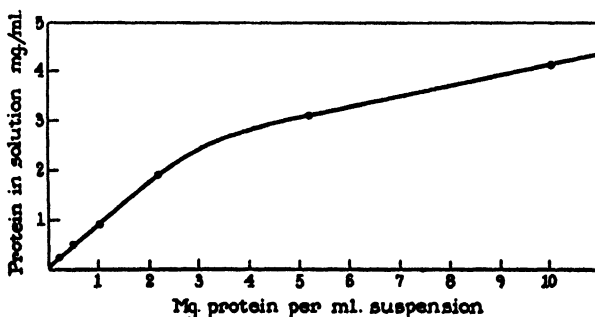


FIG. 3. Solubility of three times crystallized ricin in presence of increasing quantities of solid phase.

The concentration of protein in the filtrates was found to be as follows:

Mother liquor.....	3.0 mg./ml
First washing.....	3.1 mg./ml
Second washing.....	3.3 mg./ml

The washed crystals were resuspended in 30 ml. 0.05 M acetate buffer pH 5.5. Increasing amounts of the concentrated suspension of crystals were then pipetted into 15 ml. test tubes each provided with a pyrex glass bead. The tubes were nearly filled with the same buffer solution, stoppered with one-hole rubber stoppers, and then plugged with short glass rods. Care was taken not to leave any air bubbles in the tubes. The suspensions were revolved mechanically with a slow motion for 18 hours at about 20°C., then filtered on small No. 42 filter papers. The filtrates, as well as the suspensions before filtration, were analyzed for protein by the copper-phenol method of Herriott (4). The data are given graphically in Fig. 3. The experiment shows that the solubility of the crystals of ricin is not independent of the total amount of the excess crystals in suspension, but continues to increase gradually in the presence of

increasing amounts of the solid phase. The curve⁵ resembles the theoretical solubility curve of a solid solution of two or more components (5). Since more than one component is present it is possible that the agglutinating properties of the preparation are due to one protein and the toxic properties to another protein.

7. SUMMARY

A toxic crystalline protein has been isolated from crude extracts of castor bean meal. Ultracentrifuge and electrophoresis tests show the crystalline protein to become fairly homogeneous after three or four crystallizations. This is also confirmed by toxicity measurements. Solubility tests, however, indicate the presence of more than one protein component in the crystalline material, possibly in the form of a solid solution which cannot be separated into its components by repeated crystallization under the present technique.

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⁵ Such curves are frequently found with proteins which appear to be homogeneous by ultracentrifuge or electrophoresis methods. The constant solubility test is much more sensitive since it will detect the presence of a mixture or solid solution of proteins even though the various components have the *same* solubility. The electrophoresis or ultracentrifuge technique, on the other hand, can detect only proteins which have different rates of sedimentation or electrophoresis.

THE PRODUCTION OF A PERSISTENT ALTERATION IN INFLUENZA VIRUS BY LANTHANUM OR ULTRAVIOLET IRRADIATION

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The capacity of influenza virus to combine with and agglutinate red blood cells and thereafter to be eluted from them (1, 2) has stimulated numerous investigations. As a result of early work it was assumed that adsorption of the virus to RBC and subsequent dissociation of the complex were closely associated phenomena and it was suggested that the reactions could be considered as analogous to those between an enzyme and its substrate (2). Shortly after the discovery that influenza virus could cause hemagglutination it was found (2) that two different virus strains, *i.e.* Lee and PR8, showed different rates of elution from RBC. Recently it was shown (3) that by heating the virus the elution phenomenon could be almost completely abolished without affecting the capacity of the agent to combine with RBC and cause hemagglutination. Heat, however, inactivates the virus; the property of multiplication in susceptible hosts is lost and probably other properties also are altered. It was decided to investigate the elution of influenza virus from RBC more thoroughly and particularly to attempt to find means whereby the rate of elution of the virus could be altered without destroying the capacity of the agent to induce infection.

The results obtained in the present study provide evidence that it is possible by means of certain physical or chemical agents, *i.e.* treatment with lanthanum acetate or ultraviolet irradiation, to produce a marked alteration in the elution rate of the Lee strain of influenza virus without causing demonstrable alterations in the other properties of the virus tested. It will be shown that strains modified by the procedures employed retained their altered state on serial passage in the chick embryo in the absence of the agent which originally effected the alteration.

Materials and Methods

Viruses.—The PR8 strain (4) of influenza A virus and the Lee strain (5) of influenza B virus were used in this study. Both strains had previously been passed many times in mice and in chick embryos. Between experiments allantoic fluids infected with either virus were stored frozen in sealed ampoules in a CO₂ storage cabinet at -70°C.

Chick Embryos.—White Leghorn eggs were incubated at 39°C. for 9 to 12 days. Embryos of the desired age were inoculated into the allantoic sac through a paraffin-sealed hole drilled in the shell. The inoculum consisted of 0.1 cc. of infected allantoic fluid diluted 10⁻⁸ in sterile

broth unless otherwise stated. After inoculation chick embryos were incubated for 48 hours at 35°C. and candled daily. Embryos which died were discarded. Eggs containing living embryos then were held at 4°C. for approximately 2 hours and thereafter the allantoic fluid was harvested in the usual manner. Fluids which gave positive hemagglutination with chicken RBC were pooled and used as virus source material. Depending upon the experiment the pooled allantoic fluids were stored either at 4°C. or at -70°C.

Virus Titrations.—Hemagglutination titrations were done according to the technique described by Hirst (6). Serial twofold dilutions of allantoic fluid pools were prepared in saline buffered at pH 7.2. To 0.4 cc. of each dilution was added 0.4 cc. of a 1 per cent suspension of washed chicken RBC. The tubes were shaken and the degree of hemagglutination recorded after 1 hour at room temperature. The patterns of the sedimented erythrocytes were graded from 4+ to \pm in the customary manner and the end point was taken as the highest dilution which gave a 2+ pattern.

Virus infectivity titrations in chick embryos were done by the intra-allantoic technique (7) in 9 to 12 day embryos. Serial tenfold dilutions of allantoic fluid pools were prepared in sterile broth. A group of 4 embryos was inoculated with each dilution; each embryo received 0.1 cc. Allantoic fluid was harvested from each embryo after 48 hours' incubation at 35°C. and tested by the hemagglutination technique. The end point was taken as the highest dilution which induced demonstrable infection in 2 or more embryos in a group.

Virus infectivity titrations in mice were carried out by the intranasal technique. Serial tenfold dilutions in sterile broth were employed. A group of 6 Swiss mice was used for each dilution. The procedure was identical to that previously described (8) and the 50 per cent maximum score end point (M.S.50) was used.

Immune Serum.—Rabbits were immunized by the intravenous injection of infected allantoic fluid pools. A single injection of 10 cc. of undiluted fluid was given. Serum was obtained 14 to 21 days later and was stored without preservative at 4°C. Immediately before use in hemagglutination-inhibition tests serum was diluted 1:2 with saline and heated at 65°C. for 30 minutes. This procedure markedly reduces the so called non-specific inhibitory capacity of serum but does not significantly diminish the antibody titer of immune serum (9, 10).

Identification of Virus Strains.—Serological identification of the virus strains studied was carried out by means of the hemagglutination-inhibition technique (6). Serial twofold dilutions of infected allantoic fluid were prepared in saline. Each dilution was mixed with constant amount serum which was diluted so as to contain a quantity of antibodies just capable of inhibiting hemagglutination by 256 units of virus. To each mixture was added an equal quantity of a 1 per cent suspension of chicken RBC. The end point was taken as the highest dilution of infected allantoic fluid which caused 2+ hemagglutination. As a routine in hemagglutination-inhibition tests both anti-PR8 and anti-Lee immune as well as normal rabbit serum were employed.

Determination of Eluted Virus.—Inasmuch as the chief objective of this study was to investigate possible alterations in the capacity of influenza virus to dissociate from RBC after combination with them, it was essential to devise a technique by means of which both the rate of elution of the virus and the quantity of virus eluted could be determined. The following technique was found to be suitable: The RBC in 5 cc. of a 1 per cent suspension were packed by a few minutes' centrifugation at 4,000 R.P.M. The supernate was poured off and discarded. To the packed cells 1 cc. of an infected allantoic fluid pool was added and the mixture was shaken vigorously. Small volumes were employed to facilitate very rapid sedimentation of the RBC. Only pools with hemagglutination titers of 1:1024 or more were used. The final concentration of erythrocytes in the mixture was 5 per cent. The mixture was held at room temperature for 10 minutes in order for virus-erythrocyte combination to occur. In every instance hemagglutination occurred almost immediately after the mixture was made and the agglutinated cells settled very rapidly. At the end of 10 minutes the mixture was reshaken

and the cells sedimented by centrifugation at 4,000 R.P.M. for approximately 1 minute. The supernate was poured off and its hemagglutination titer determined subsequently. Immediately 1 cc. of buffered saline was added to the packed cells; this mixture was shaken vigorously and then held at room temperature. Identical cycles of centrifugation, removal of supernate, and resuspension of the sedimented cells in 1 cc. of fresh saline were carried out 30, 60, 90, 120, and 180 minutes later. Each supernate was kept so that its hemagglutination titer could be determined. In certain experiments even shorter periods were employed in each successive elution step.

This technique has made it possible to obtain fairly reproducible virus elution curves relative to time and has facilitated the present study. It should be pointed out that the resuspension of the agglutinated cells in fresh saline after each centrifugation makes it possible to determine the dissociation of relatively small quantities of virus during each time interval which is technically very difficult if the initial cell-virus mixture is retained and supernate aliquots are removed successively. It should be mentioned also that it is important to employ a relatively large amount of virus so that all RBC "receptors" rapidly combine with the agent; *i.e.*, are saturated. Otherwise, virus particles which dissociate from one RBC could promptly combine with another and only appear in the supernate subsequently thus leading to an inexact estimation of elution rate. It was found that under the conditions of these experiments allantoic fluid pools with hemagglutination titers of 1:1024 or more consistently saturated the quantity of cells employed as judged by the finding that demonstrable amounts of virus remained unadsorbed 10 minutes after the mixtures were prepared.

Ultraviolet Irradiation.—In all experiments the same ultraviolet source and distance were used. The lamp, which was kindly provided by Dr. George I. Lavin, was a "cold arc" resonance mercury lamp which operated at 15,000 volts A.C. The emission spectrum showed one main line at 2537 Å. Irradiation of infected allantoic fluid pools was performed in an open Petri dish with an inner diameter of 5 cm. Five cc. of fluid in this dish gave a depth of 5 mm. The distance from the fluid to the disc-shaped coil (diameter = 8 cm.) of the lamp, which was parallel to the fluid surface, was 9 cm. Irradiation was carried out for periods ranging from 15 to 60 minutes. Allantoic fluids were not dialyzed before irradiation.

Treatment with Lanthanum.—Lanthanum acetate (LaAc_3) was used for the treatment of infected allantoic fluid pools. The amorphous compound was dissolved in distilled water. Two stock concentrations, 0.1 and 0.01 M, were employed and the pH of the solutions used varied only between 6.5 and 7.0. When tested intra-allantoically in chick embryos, it was found that injections of 0.5 cc. of 0.1 M solutions did not kill the embryos. Solutions of LaAc_3 were mixed with equal quantities of infected allantoic fluid pools so as to give final concentrations ranging from 0.005 to 0.00005 M. The mixtures were held at 4°C. for 3 to 5 hours. Flocculent precipitates developed promptly. The precipitate was removed by centrifugation and discarded. The supernate was employed and excess lanthanum contained in it was precipitated by the addition of sufficient Na_2HPO_4 to give 0.5 M. This was done because lanthanum acetate even in very low concentrations causes agglutination of chicken RBC.

EXPERIMENTAL

Elution of Lee and PR8 Strains from RBC.—In order to determine whether persistent alterations in the rate of elution of influenza virus from RBC could be produced by experimental procedures, it was first necessary to devise a technique by means of which relatively small quantities of eluted virus could be measured. It was found that, when the RBC were sedimented from the mixture at fixed time intervals and resuspended in fresh saline after each centrif-

ugation, it was possible to measure with fair reproducibility the amount of virus which was eluted during each successive time interval. It appeared that in this manner the information desired could be obtained.

A number of experiments were carried out to determine the rates of elution of the Lee and PR8 strains from chicken RBC under these conditions. Hirst (2) showed previously, under different experimental conditions, that the Lee strain was eluted more rapidly than the PR8 strain.

The stepwise technique employed for the determination of the rate of elution of influenza viruses from RBC is described in detail above. In the present experiments successive 30 minute elution intervals were employed.

The results of a series of such experiments with a number of allantoic fluid pools containing either the Lee or the PR8 strain are presented in summary form in Table I. It can be seen that elution of the Lee strain is characterized by very rapid dissociation during the first 30 minute step and thereafter during successive 30 minute intervals by progressively less rapid dissociation. It is evident that after the second step (70 minutes) only very small quantities of virus were released into the supernatant fluids. The progressively decreasing rate of elution becomes especially evident when the percentage of virus eluted during each step is calculated relative to that combined with the RBC at the beginning of each step. As is shown in Table I, this quantity diminishes rapidly from an average value of 27.3 per cent during the first 30 minute step to no more than 0.3 per cent during the final 60 minute step.

The elution of the PR8 strain showed a very different pattern. Only relatively small amounts of virus were released from the RBC during each 30 minute interval and at no time did the titer of the supernates reach a high level as was the case with the Lee strain during the first 30 minute step. Moreover, it will be noted that the percentage of combined virus which was eluted during each step remained almost constant. This indicates that with the PR8 strain the rate of elution did not vary significantly during the time interval studied. In Figs. 1 and 2 the amounts of each strain which were eluted in successive steps are presented graphically. The logarithms of the geometric mean of the hemagglutination titers of the supernates are plotted against the time after the virus was mixed with RBC.

Similar experiments in which the supernatant fluid was changed at shorter intervals were carried out with both the Lee and PR8 strains. In every instance analogous results were obtained. Although it was possible by employing very short elution periods, *e.g.* 5 minutes or less, to obtain even more rapid elution rates with the Lee strain, it was not possible to increase the rate of elution of the PR8 strain as can be seen from the data shown in Fig. 3.

It is apparent that a number of factors might be responsible for the results obtained with the Lee strain. However, in the light of the findings presented

below, it seems of most interest to consider the possibility that the progressively decreasing rate of elution obtained with the Lee strain as not with PR8, may be an indication of an inhomogeneous population of Lee virus particles in infected allantoic fluid.

Effects of Lanthanum Acetate upon Influenza Viruses.—Hammarsten, Hammarsten, and Teorell (11) showed that lanthanum reacts with nucleic acids to

TABLE I
Results of Stepwise Virus Elution Experiments with Lee and PR8 Strains

Virus strain	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer* of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	9	min. 0 (Control)	8192	1024	2757	per cent —
"	"	10	256	16	34	—
"	"	40	2048	256	745	27.3
"	"	70	512	64	161	8.1
"	"	100	256	16	32	1.7
"	"	130	32	4	10	0.5
"	8	190	8	4	5	0.3
Total virus eluted.....					953	37.9
PR8	5	0 (Control)	4096	1024	2665	—
"	"	10	32	16	28	—
"	"	40	128	32	64	2.4
"	"	70	128	64	112	4.3
"	"	100	256	32	74	3.0
"	"	130	128	32	56	2.3
"	"	190	128	32	97	4.2
Total virus eluted.....					403	16.2

* Expressed as the reciprocal.

form an insoluble complex. It seemed possible that lanthanum could also react with influenza viruses and probable that, if it did, it might cause some alteration of the agents. This possibility was investigated.

When an aqueous solution of lanthanum acetate was added to normal allantoic fluid, a flocculent precipitate developed. The more concentrated the LaAc₃ solution, the more abundant was the precipitate. It was found that even in high dilution, e.g. 10^{-6} to 10^{-8} M, LaAc₃ caused agglutination of chicken RBC. Consequently, it was impossible to determine the hemagglutination titer of the virus in the presence of free lanthanum. When, however, a few

drops of 5 M Na_2HPO_4 solution were added to the mixture, the excess of LaAc_3 was precipitated and satisfactory virus hemagglutination titrations could be performed.

The addition of LaAc_3 to allantoic fluids containing influenza virus caused a prompt reduction in the hemagglutination titer of the virus and the reduction in titer appeared to be proportional to the concentration of lanthanum. Thus, a concentration of 0.0001 M did not

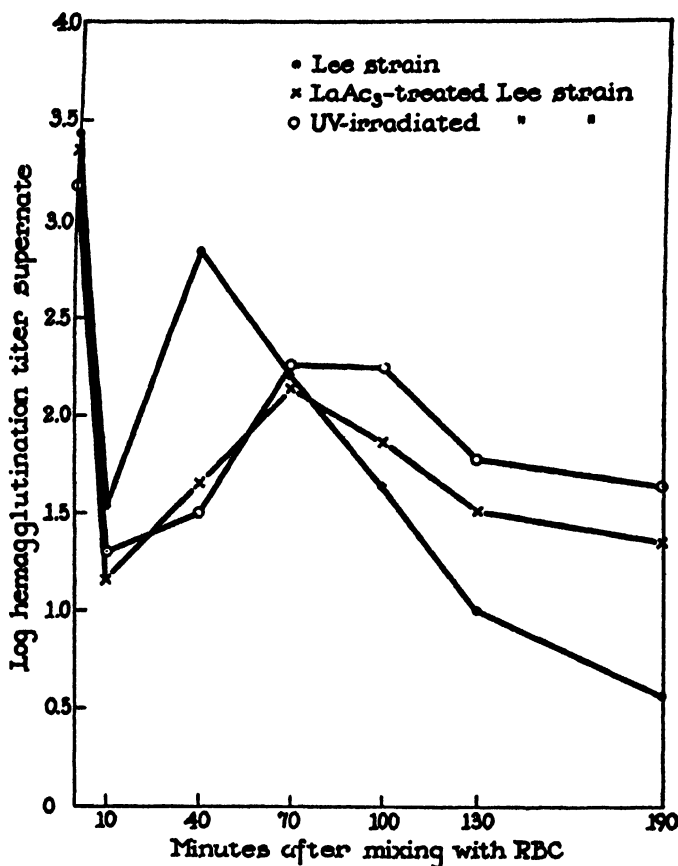


FIG. 1. Stepwise elution curves obtained with the Lee strain as well as with LaAc_3 -treated and ultraviolet-irradiated strains derived therefrom. The logarithm of the geometric mean of the hemagglutination titers of successive supernates (*cf.* Tables I, II, and IV) is plotted against the time at which the supernate was removed.

demonstrably affect the hemagglutination titer, concentrations of 0.01 to 0.001 M decreased the titer 100- to 1000-fold, while a concentration of 0.1 M abolished the hemagglutination reaction completely. The reduction in titer occurred immediately after the lanthanum was added and prolonged treatment, *e.g.* 10 hours, did not lead to any further decrease in titer.

The effect of LaAc_3 upon the infectivity of influenza virus also was studied. It was found that a mixture of equal parts of allantoic fluid infected with the

Lee strain and a 0.01 M solution of LaAc_3 was capable of infecting chick embryos. The virus infectivity titer of such a mixture, however, was lower by about 3 logarithmic units than that of the untreated allantoic fluid. Prolonged

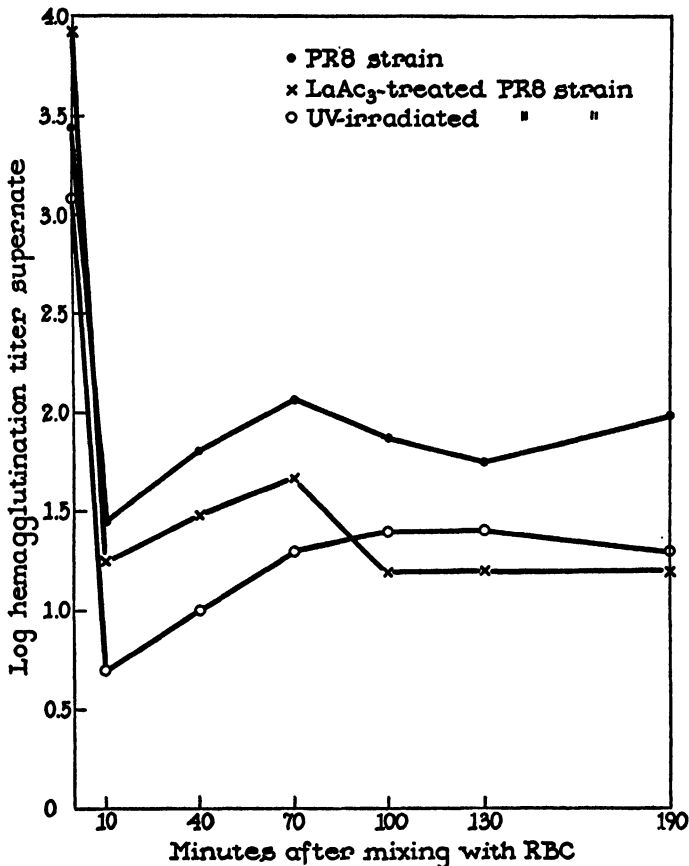


FIG. 2. Stepwise elution curves obtained with the PR8 strain as well as with LaAc_3 -treated and ultraviolet-irradiated strains derived therefrom. The logarithm of the geometric mean of the hemagglutination titers of successive supernates (*cf.* Tables I, II, and IV) is plotted against the time at which the supernate was removed.

contact, *e.g.* 8 hours, with LaAc_3 did not cause any further decrease in the virus infectivity titer.

Elution of Lanthanum-Treated Virus from RBC.—To determine whether treatment with LaAc_3 caused an alteration in the rate of elution of the Lee or PR8 strains was technically difficult with most of the original mixtures because of their markedly reduced hemagglutination titers. However, when a concentration of 0.00025 M was employed with the Lee strain, the hemagglutination

titer was reduced only two- to fourfold and it was possible to demonstrate directly that elution of this treated strain occurred at a definitely slower rate than that of the original strain.

Because virus treated with LaAc_3 retained infectivity for the chick embryo, it was of obvious interest to determine the rate of elution of lanthanum-treated virus from RBC after serial passage in the allantoic sac. A number of experiments were carried out with both the Lee and PR8 strains.

Allantoic fluid pools infected with the desired virus were mixed with LaAc_3 solution so as to yield a concentration of 0.005 M. After 3 to 5 hours at 4°C . and light centrifugation to

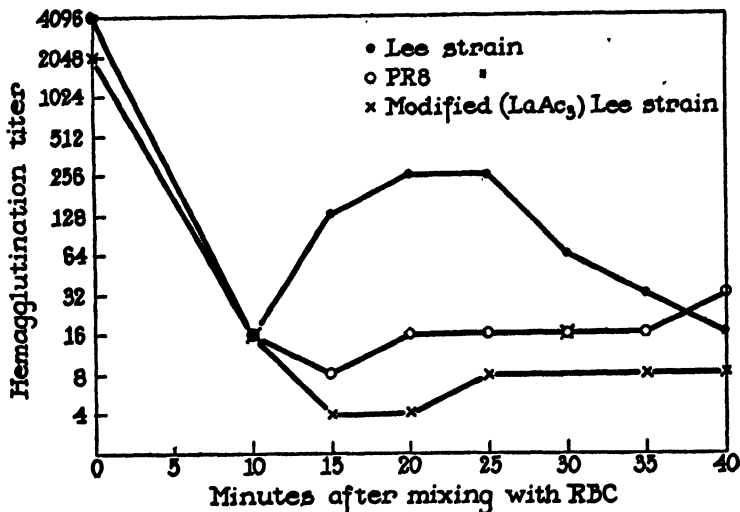


FIG. 3. Stepwise elution curves obtained by removal of the supernate at 5 minute intervals with the Lee and PR8 strains as well as a modified (LaAc_3 -treated) Lee strain.

remove the precipitate, groups of 6 embryos were inoculated intra-allantoically with a 10^{-2} or 10^{-3} dilution of each supernate. The embryos were incubated and their allantoic fluids removed as described above. All fluids from each group which gave positive hemagglutination tests were pooled and used either in elution rate experiments or as inocula for additional groups of embryos. In passages the inoculum was diluted 10^{-3} . No further treatment with LaAc_3 was employed during the serial passage experiments. After a single treatment with LaAc_3 the Lee strain was carried through 15 serial passages in the allantoic sac; the PR8 strain through 7.

The results of stepwise elution rate experiments with LaAc_3 -treated strains of virus after serial passage in the chick embryo are shown in Table II as well as in Figs. 1 and 2. With the Lee strain the allantoic fluid pools employed ranged from the 1st through the 15th serial passage. With the PR8 strain pools obtained at each passage from the 1st through the 7th were used. It

is seen that the rate of elution of the LaAc₃-treated Lee strain from RBC was strikingly different from that of the original Lee strain (*cf.* Table I). The very rapid elution rate during the first 30 minute step was no longer demonstrable and the progressive decrease in the elution rate, which characterized the original strain, was no longer evident. Moreover, the total quantity of virus which was eluted during successive steps was approximately $\frac{1}{8}$ of the

TABLE II

Results of Stepwise Elution Experiments with Lanthanum-Treated Lee and PR8 Strains after Serial Passage in the Chick Embryo

Virus strain (LaAc ₃ -treated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	9	min. 0 (Control)	8192	1024	2352	per cent —
"	"	10	128	4	15	—
"	"	40	512	4	35	1.4
"	"	70	512	8	138	5.6
"	8	100	256	32	70	3.0
"	9	130	128	8	32	1.4
"	"	190	64	8	24	1.1
Total virus eluted					299	12.5
PR8	7	0 (Control)	4096	1024	2470	—
"	"	10	64	8	18	—
"	"	40	512	8	24	1.0
"	"	70	256	16	29	1.2
"	"	100	256	16	35	1.5
"	"	130	128	16	39	1.6
"	"	190	128	16	39	1.7
Total virus eluted					166	7.0

amount obtained with the original strain. In these respects, the elution of the LaAc₃-treated Lee strain closely resembled that of the original PR8 strain (*cf.* Table I). It seems important to point out that prolonged serial passage with infected allantoic fluid inocula diluted only 10^{-8} , did not cause reversion in the peculiar elution characteristics of the LaAc₃-treated Lee strain; after 15 passages in the absence of lanthanum, the elution curve remained grossly abnormal and was not significantly different from that obtained after but a single passage of the modified strain in the allantoic sac.

It also is seen from the results shown in Table II that the rate of elution of

the LaAc₃-treated PR8 strain from RBC was somewhat slower than that of the original PR8 strain. It should be noted, too, that only about $\frac{1}{2}$ as much of the treated strain as of the original strain was eluted during successive elution steps in the time period studied. Even after 7 serial passages in the allantoic sac in the absence of lanthanum these alterations in the elution curve of the LaAc₃-treated PR8 strain remained demonstrable; no evidence for reversion to the characteristics of the original strain was obtained.

Elution of Ultraviolet-Irradiated Virus from RBC.—Henle and Henle (12) showed that ultraviolet irradiation inactivated various potentialities of influenza virus at different rates. Under the experimental conditions which they employed there was but little effect upon elution of the virus unless irradiation was prolonged sufficiently to affect markedly the capacity of the virus to cause hemagglutination.

In the light of the unexpected and apparently persistent modifications produced by a single treatment with LaAc₃ in the strains under study, it appeared of interest to study further the effects of ultraviolet irradiation upon the Lee and PR8 strains.

Undiluted allantoic fluid pools infected with the desired virus were irradiated with high intensity ultraviolet for varying periods under the conditions described above. After irradiation the hemagglutination titers of the fluids were determined. The longer periods (45 to 60 minutes) of irradiation employed caused a marked reduction in the hemagglutination titer of previously non-irradiated strains. Irradiated fluids were diluted 10^{-8} , and each was inoculated intra-allantoically into a group of 6 embryos. The embryos were incubated and their allantoic fluids harvested as described above. These allantoic fluids were used in elution rate experiments as well as for the inoculation of additional groups of embryos. In passages the inoculum was diluted 10^{-8} . With two irradiated Lee strains 6 serial passages of each, after a single period of irradiation, were carried out in the allantoic sac. With another irradiated Lee strain 11 serial passages were performed and several additional irradiations were carried out between certain passages. With the PR8 strain 7 serial passages were performed and further irradiation was carried out between each passage.

The results of stepwise elution rate experiments with two ultraviolet-irradiated Lee strains after serial passage in the chick embryo are shown in Table III. With these two strains, which were irradiated only once, allantoic fluid obtained after each passage from the 2nd through the 6th was studied. It will be noted that the elution rate of these two irradiated Lee strains was markedly slower than that of the original Lee strain (*cf.* Table I) even though the total amount of virus eluted during all the successive steps was not greatly different. As with the LaAc₃-treated Lee strain (*cf.* Table II), the most striking reduction was demonstrated during the first 30 minute elution period.

The results of similar experiments with a repeatedly irradiated Lee strain are shown in Table IV and in Fig. 1. These experiments were made in an attempt to reduce even more markedly the elution rate of the irradiated Lee strain by means of further irradiation of allantoic fluid between embryo pas-

TABLE III

Results of Stepwise Elution Experiments with Lee Strains after a Single Ultraviolet Irradiation and Serial Passage in the Chick Embryo

Virus strain (ultraviolet- irradiated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	10	min. 0 (Control)	8194	1024	1684	per cent —
"	"	10	256	16	42	—
"	"	40	1024	4	73	4.4
"	"	70	1024	16	157	10.0
"	"	100	256	32	91	6.4
"	"	130	128	8	45	3.4
"	"	190	256	4	42	3.3
Total virus eluted					408	27.5

TABLE IV

Results of Stepwise Elution Experiments with Lee and PR8 Strains Repeatedly Ultraviolet-Irradiated after Serial Passage in the Chick Embryo

Virus strain (ultraviolet- irradiated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	10	min. 0 (Control)	2048	1024	1520	per cent —
"	"	10	128	0	21	—
"	"	40	128	16	32	2.1
"	"	70	512	64	181	12.3
"	"	100	512	32	179	13.9
"	"	130	256	16	60	5.4
"	"	190	128	8	42	4.0
Total virus eluted					494	37.7
PR8	7	0 (Control)	4096	1024	2030	—
"	"	10	32	0	5	—
"	"	40	32	0	10	0.5
"	"	70	64	4	20	1.0
"	"	100	128	8	26	1.3
"	"	130	64	8	26	1.3
"	"	190	64	8	20	1.0
Total virus eluted					102	5.1

sages. It is evident that the altered elution rate of the irradiated Lee strain was not additionally affected by further irradiation.

It can be seen also from the results shown in Table IV and Fig. 2 that the elution rate of a repeatedly irradiated PR8 strain was somewhat slower than that of the original PR8 strain (*cf.* Table I) and that only approximately $\frac{1}{3}$ as much of the irradiated strain as of the original strain was eluted during the time interval studied. The elution rate of the irradiated PR8 strain corresponded very closely to that of the LaAc₃-treated PR8 strain (*cf.* Table II).

With the irradiated Lee strains no evidence of reversion to the elution characteristics of the original strain was obtained despite serial passage in the embryo with no further irradiation. The elution rates obtained with fluids from the last serial passages, *i.e.* the 6th, appeared not to differ significantly from those obtained with fluids from the 2nd embryo passages. It appears, therefore, that the modifications induced by ultraviolet irradiation, with respect to elution of the strains studied from RBC, persisted on serial passage in the absence of the original agent, as was the case after treatment with LaAc₃.

Infectivity of Modified Strains.—After serial passage in the chick embryo the infectivity titer of LaAc₃-treated Lee and PR8 strains as well as ultraviolet-irradiated Lee and PR8 strains was determined in parallel with that of the original strains. Titrations were carried out both in mice and in chick embryos according to the techniques described above.

In every instance it was found that there was no significant difference between the virus titration end points obtained with the modified and original strains in either mice or chick embryos. It appears, therefore, that both LaAc₃-treated and ultraviolet-irradiated strains retained in undiminished degree the capacity to induce infection in either species.

Immunological Properties of Treated Strains.—After serial passage in the chick embryo cross hemagglutination-inhibition titrations were carried out with LaAc₃-treated, ultraviolet-irradiated, and the original Lee and PR8 strains. The immune sera employed were anti-Lee, anti-PR8, and anti-ultraviolet-irradiated Lee.

In each case it was found that there was no significant difference between the results of quantitative hemagglutination-inhibition titrations with the original strain and with modified strains derived from it. It thus appears that both the LaAc₃-treated and the ultraviolet-irradiated strains retained the immunological specificity which characterizes the strain from which they were derived.

Effect of Increased NaCl Concentration upon Elution Rate.—In this laboratory Davenport (13) recently found that a decrease of electrolyte concentration in RBC-influenza virus systems leads to a reduction in the elution rate of the virus. In the present study, when the NaCl concentration of mixtures was reduced in experiments in which the stepwise elution technique was employed,

a decrease in elution rate also was noted. It was, therefore, of interest to determine what effect an increased salt concentration would have upon the elution rate under similar conditions.

TABLE V

Results of Stepwise Elution Experiments with Lee, PR8, and Ultraviolet-Irradiated Lee Strains in 3 Per Cent NaCl Solution

Virus strain	Time after mixing with RBC	Hemagglutination with successive supernates* diluted as indicated												Total virus eluted in	
		4	8	16	32	64	128	256	512	1024	2048	4096	3.0 per cent NaCl	0.9 per cent NaCl	
Lee	min.														
	0 (Control)	2	2	2	3	3	3	3	3	2	2	2			
	10	2	2	3	2										
	40	0	0	±	2	2	2	2	3	2	2	2			
	70	3	3	3	3	2	2								
	100	3	2	2											
	130	2													
190	2											104	55		
Ultraviolet-irradiated Lee	0 (Control)	4	4	4	3	3	3	3	3	3	2	2			
“ “	10	3	3	2											
“ “	40	4	3	3	3	2									
“ “	70	4	4	3	3	3	2								
“ “	100	4	3	3	3	3	2								
“ “	130	3	3	3	3	2									
“ “	190	3	3	3	3	2							11	13	
PR8	0 (Control)	4	4	4	3	3	3	3	3	3	2	2			
“	10	4	3	2											
“	40	4	4	4	4	4	4	3	2						
“	70	4	4	4	4	3	3	2	2						
“	100	4	4	4	4	3	3	2	2						
“	130	4	4	3	3	2	2	2							
“	190	4	4	4	3	3	2	2					50	10	

* Dilutions prepared in 0.9 per cent NaCl solution.

A buffered solution containing 3 per cent NaCl, instead of 0.85 per cent, was used. When this solution was added to sedimented RBC and adsorbed virus, it was found that the RBC promptly formed a solid gel. If, however, the RBC were first gradually adapted to an increasing salt concentration by washing successively in 1.5, 2, 2.5, and 3 per cent NaCl solutions, no such difficulty was encountered. The experiment, therefore, was carried out with such NaCl-adapted RBC and 3 per cent buffered NaCl solution was added as an elution medium at each step. The supernates were diluted in 0.9 per cent NaCl in the usual manner.

As can be seen from the results shown in Table V the presence of 3 per cent NaCl in the mixtures increased the elution rate of both the Lee and PR8 strains. However, the modified (ultraviolet-irradiated) Lee strain did not show any significant change in elution rate under these conditions. It should also be noted that in the lower dilutions of the supernate obtained from the Lee strain after the first 30 minute elution step there was no evidence of hemagglutination at 1 hour. With the other supernates from the Lee strain as well as with each of the supernates from the PR8 and the modified Lee strain, the usual results were obtained.

Simultaneous hemagglutination titrations were performed with the Lee and PR8 strains in 3, 2, 0.9, and 0.45 per cent, respectively, buffered NaCl solutions. In 3 per cent NaCl solution the Lee strain causes agglutination rapidly, but after about 25 minutes aggregates disappeared, and at the end of 1 hour only 0 readings were obtained throughout the series of dilutions. In 2 per cent NaCl solution the lowest four dilutions showed 0 readings. No similar disappearance of hemagglutination occurred in either 0.9 or 0.45 per cent NaCl solutions. Identical experiments with the PR8 strain failed to show any such effect of NaCl concentration on the hemagglutination reaction.

It appears that it was possible to increase the elution rate of both the Lee and PR8 strains but not of the modified Lee strain by increasing the NaCl concentration of the elution medium. The disappearance of hemagglutination in the lower dilutions of the supernate from the first 30 minute step is undoubtedly the result of the very rapid elution rate of this fraction which is further accelerated by the increased NaCl concentration. It is of interest that both the PR8 strain and the modified Lee strain, both of which have relatively slow elution rates, failed to show a similar phenomenon.

DISCUSSION

That it is possible to cause certain alterations in a property of influenza virus by well defined and readily controlled laboratory procedures is apparent from the results obtained in this study. A single brief period of treatment with lanthanum acetate in low concentration or a single period of irradiation with ultraviolet light resulted in the development of virus strains which possessed a demonstrable modification as compared with the original strain. Of most interest and importance is the fact that the modified strains retained their unusual character on serial passage in the chick embryo in the absence of the agent which originally produced the alteration.

Insofar as was determined but a single property of the modified strains was altered. That other properties also may have been altered is, of course, possible. However, infectivity for both the mouse and the chick embryo, hemagglutinating capacity, rate and degree of adsorption on RBC, antigenicity and immunological specificity all appeared to be identical with those of the original

strain. Of the properties studied only elution from combination with RBC was demonstrably different, both as to rate and extent, and served to distinguish the modified strains of Lee virus from the original strain.

It is noteworthy that it is the Lee strain which yielded modified strains with the more striking reductions in elution rate. It has been known for some time (2) that the Lee strain elutes from RBC considerably more rapidly than the PR8 strain. The alteration was sufficiently marked to make it impossible to distinguish between modified Lee strains and the PR8 strain on the basis of their rates of elution alone.

As for the agents which were employed for the development of modified influenza virus, it is known that lanthanum forms insoluble complexes with nucleic acids (14) and the wave length of ultraviolet employed appears to be identical with that at which nucleic acids show maximal adsorption (15). Whether either or both agents exert their effects as a result of action on nucleic acid components of the virus particles is hypothetical. However, it appears possible that either agent might deleteriously affect such components.

The fact that the original Lee strain shows a progressively decreasing rate of elution with increasing time suggests that the individual virus particles are not entirely identical as regards this one property. There is, in fact, no good reason for thinking that all virus particles of a given strain are identical in all respects. There is some evidence which strongly suggests that influenza viruses, like numerous other infectious agents, may show variation relative to various properties; *i.e.*, pathogenicity for unnatural hosts (16, 17), capacity to agglutinate erythrocytes from various species (18), and immunological characteristics (19, 20). It seems probable that an infected allantoic fluid can be considered to contain an enormous population of virus particles all fundamentally similar in most respects but differing slightly, one from another, in certain respects. If such an inhomogeneity in a given population of virus particles exists, it would not be surprising that chemical or physical agents might affect individual particles in somewhat different ways. Thus, the deleterious action of lanthanum or of ultraviolet might vary in an inhomogeneous population, and those particles which, following treatment, retained the capacity to multiply in a susceptible host would be in all probability the particles least affected by either agent. Passage of such a treated strain should result in the development of a virus population closely similar to or identical, in distribution of properties, with that of the original strain only if the treated strain were as capable as the original strain of leading to the development of virus particles with slightly diverse properties. If, however, a treated strain contains less than the original distribution of slightly different virus particles, and leads in the susceptible host to the development of a virus population with an abnormally restricted distribution of properties, an altered strain would emerge. Such an altered strain could continue on serial passage to show the same unusual

property, although reversion with the eventual acquisition of the properties of the original strain might occur if passages were continued sufficiently long.

It seems likely that the alteration produced in influenza virus in the present study is best explained on the basis of selection of naturally occurring variants. It is, of course, possible that variants might actually have been induced by the procedures employed. With the modified Lee strains it appears that the relatively large proportion of the virus population of the original strain which shows a very rapid elution rate, is much reduced. The slower elution rate of the modified strains may be explained by the reproduction in the susceptible host of a higher than normal proportion of virus particles with a slow elution rate due presumably to the selective inactivation of virus particles with a high elution rate by the agents initially employed. Whether variants were selected or induced by the experimental procedures, the final results would be similar and a modified strain could be evolved under either circumstance.

SUMMARY

The rates of elution from RBC of the Lee and PR8 strains of influenza virus were studied by means of a step-wise elution technique. By means of a single treatment with lanthanum acetate or irradiation with ultraviolet and subsequent passage in chick embryos, it was possible to alter the elution rate of the Lee strain so that it was similar to that of the PR8 strain. This alteration proved to be persistent on serial passage in the absence of the agent which caused it. As far as was determined, the elution rate of the virus appeared to be the only property which was altered. The phenomenon can be most readily understood on the assumption that the difference in elution rates of the two strains is due to a heterogeneous population of virus particles in the Lee strain with respect to elution rate.

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REACTIONS BETWEEN INFLUENZA VIRUS AND A COMPONENT OF ALLANTOIC FLUID

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It is well known that there are present in certain tissues of various normal animal species components which are capable of inhibiting hemagglutination by influenza viruses (1-10). Relatively little attention has been given to a component of this kind which is present in the allantoic fluid of normal chick embryos. Svedmyr (7) previously reported upon such a component and studied some of its properties. In view of the fact that, at the present time, most work on influenza viruses is carried out with material obtained from the allantoic sac of the chick embryo, it seemed important to study in detail this inhibitor and the reactions between it and influenza viruses. In the present communication evidence will be presented regarding its nature. Evidence also will be presented to show that it combines with the virus particle, and that following combination the complex dissociates partially, but not completely. In addition, it will be shown that after dissociation the inhibitor is altered, *i.e.* it is no longer capable of combining with additional influenza virus, and therefore cannot be demonstrated directly by the available techniques.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was used. The virus, which previously had been passed many times through chick embryos and mice, was cultivated in the allantoic sac of White Leghorn chick embryos which had been incubated at 39°C. for 10 to 12 days. After inoculation with approximately 10^6 E.I.D. of virus the eggs were incubated at 35°C. for 48 hours, and then chilled at 4°C. overnight before the allantoic fluids were harvested. The infected allantoic fluids employed in hemagglutination experiments were sterile pools obtained from groups of 8 to 10 eggs and were stored at 4°C. Infected fluids which were used for the inoculation of additional eggs were diluted with nine parts of sterile normal horse serum (previously heated at 56°C. for 30 minutes) and stored in nitrocellulose tubes at -70°C.

Normal Allantoic Fluid.—For the most part the normal allantoic fluid used was removed from chick embryos which had been incubated at 39°C. for 14 days. Following incubation the eggs were chilled at 4°C. overnight and the allantoic fluid from groups of 8 to 10 eggs was pooled. Each pool of normal fluid was stored at 4°C. until used. Frequently, a precipitate formed in the allantoic fluid upon storage at 4°C.; this was removed by centrifugation and discarded.

Virus Hemagglutination Titrations.—Serial twofold dilutions of infected allantoic fluid were made in 0.85 per cent NaCl solution buffered at pH 7.2 (0.01 M phosphate). To 0.4 cc. of each dilution in 10 × 75 mm. Pyrex tubes was added 0.4 cc. of a 0.5 per cent suspension of washed chicken RBC in buffered saline. Readings were made after the tubes had stood 1 hour at room temperature. The end point was taken as the highest dilution at which definite (2+) agglutination of the RBC occurred.

EXPERIMENTAL

Increase in the Inhibitor in Extraembryonic Fluids with Age.—Before undertaking a detailed study of the component in normal allantoic or amniotic fluid which inhibits hemagglutination by influenza virus, it was first necessary to determine the age of the embryo at which the fluids contained most inhibitor.

Groups of 10 to 20 normal embryonated eggs were incubated at 39°C. for periods from 7 to 14 days. After incubation the allantoic and amniotic fluids from each group of eggs were pooled separately and each pool was tested for its ability to inhibit hemagglutination with the PR8 strain. Two methods were used: With Method I serial twofold dilutions of a single virus suspension were made in each allantoic or amniotic fluid pool, and the hemagglutination titers obtained were compared with the titer of the virus diluted in buffered saline. With Method II serial twofold dilutions of each pool of allantoic or amniotic fluid were made in buffered saline; to each dilution was added a suspension of heated virus (56°C. for 1 hour) diluted so as to give a final concentration of 4 hemagglutinating units. The titer of the inhibitor was taken as the highest dilution of fluid which prevented definite (1+) hemagglutination.

The results of the experiments with allantoic fluid are presented in Fig. 1 A. The curve obtained by Method I is plotted as the difference between the logarithms of the hemagglutination titers obtained when the virus was diluted in normal allantoic fluid as compared to buffered saline. The curve obtained by Method II is plotted as the logarithm of the highest dilution of normal allantoic fluid which prevented hemagglutination by 4 units of virus. It will be seen that both methods gave similar results, although the curve obtained by Method II has a slightly greater slope. The results demonstrate that the concentration of inhibitor in normal allantoic fluid increases progressively with increasing age of the embryo. In general these results are in agreement with the findings of Svedmyr (7) who employed a technique analogous to Method I. However, the present results do not indicate that the concentration of inhibitor is relatively constant after the 11th or 12th day, as was previously suggested (7).

The results of experiments with amniotic fluid are shown in Fig. 1 B in which the plotting procedure is identical with that employed in Fig. 1 A. Here again, the slope obtained by Method II is greater than that obtained by Method I. It will be noted that there is almost no demonstrable inhibitor in amniotic fluids from normal eggs less than 10 days of age, but that thereafter the concentration of inhibitor increases rapidly with increasing age of the embryo until a peak is reached at 13 days. The inhibitor concentration as determined by Method II at this time is greater than that of allantoic fluid from 14 day embryos. These findings are similar to those of Svedmyr (7) insofar as they may be compared.

Determination of Inhibitor Concentration.—The methods employed to determine the concentration of inhibitor in normal allantoic fluid were of two main types: (a) inhibition of decreasing quantities of virus by a constant quantity

of inhibitor, and (b) inhibition of a constant quantity of virus by decreasing quantities of inhibitor. They are presented in detail below.

Decreasing Virus versus Constant Inhibitor.—In the early experiments an infected allantoic fluid pool was diluted serially in normal allantoic fluid and the hemagglutination titer obtained in the usual manner was compared with the titer obtained after dilution of the same pool in

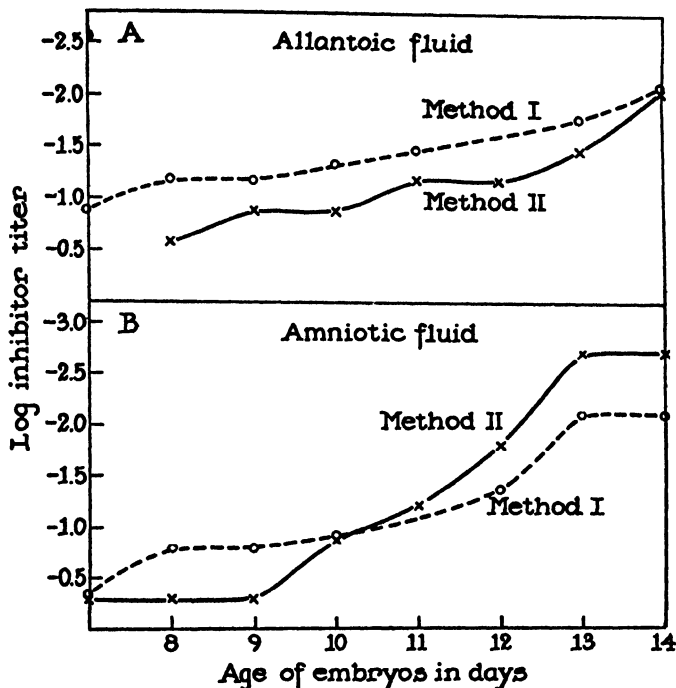


FIG. 1. Increase in inhibitor concentration in extraembryonic fluids with age. Graph A represents results obtained with normal allantoic fluid. Graph B shows the results obtained with normal amniotic fluid. The concentration of inhibitor was determined by two methods: In Method I the virus was diluted in allantoic or amniotic fluid and the log of the difference in titer from a control titration in saline is shown; in Method II dilutions of the fluids were tested against 4 units of heated virus and the log of the highest dilution which inhibited hemagglutination is shown.

buffered saline. In this procedure it is evident that the concentration of normal allantoic fluid increases slightly but progressively with each dilution of the infected pool. In order to control this variable the following procedure was employed.

Serial twofold dilutions of an infected pool were made in buffered saline. To 0.2 cc. of each dilution were added 0.2 cc. of normal allantoic fluid from 14 day old embryos and 0.4 cc. of a 0.5 per cent suspension of washed chicken RBC. The hemagglutination titer obtained was compared with that obtained in a similar titration in which buffered saline was substituted for normal allantoic fluid. The number of hemagglutinating units of virus inhibited was taken as the ratio of the titer in saline to the titer in the presence of normal allantoic fluid.

In Table I the results obtained in a number of experiments carried out by this method are summarized. It will be seen that between 1 and 24 hemagglutinating units of virus were inhibited by normal allantoic fluid when different pools of virus were employed. However, in each instance the quantity of virus inhibited was directly related to the titer of the virus pool used. That is, in the presence of a constant concentration of normal allantoic fluid an almost constant hemagglutination titer is obtained regardless of the level of the original virus titer. Therefore, in order to determine differences in the concentration of inhibitor, it is important to use a virus pool with the highest possible titer.

TABLE I

Relation between Hemagglutination Titer and Quantity of Virus Inhibited by a Constant Amount of Normal Allantoic Fluid

Infected* allantoic fluid pool	Hemagglutination titer†		Virus inhibited
	In buffered saline	In normal allantoic fluid‡	No. of hemagglutinating units
A	256	128	1
B	512	128	2
C	1024	128	4
D	2048	128	8
E	2048	64	16
F	6144	128	24

* Allantoic fluids harvested 48 hours after inoculation of PR8.

† Expressed as the reciprocal.

‡ Obtained from 14 day embryos.

Constant Virus versus Decreasing Inhibitor.—In this procedure serial twofold dilutions of normal allantoic fluid from 14 day embryos were made in buffered saline. To 0.4 cc. of each dilution were added 0.2 cc. of virus, diluted so as to give a final concentration of 4 hemagglutinating units, and 0.2 cc. of a 1 per cent suspension of washed chicken RBC. The titer of the inhibitor was taken as the highest dilution of normal allantoic fluid which prevented definite (1+) hemagglutination.

The results of a typical experiment are shown in Table II. It is seen that normal allantoic fluid inhibited hemagglutination only when it was diluted eightfold or less. Of numerous fluids tested none could be diluted more than 1:8 and still cause inhibition under these conditions.

Constant Heated Virus versus Decreasing Inhibitor.—It was shown by Francis (5) that influenza B virus, when heated at 56°C., lost very little of its hemagglutinating capacity, but gave much higher hemagglutination inhibition titers with normal sera than did equivalent amounts of unheated virus. It seemed possible that similar results might be obtained with normal allantoic fluid, and

consequently titrations were carried out in a manner identical with that described above except that 4 hemagglutinating units of heated virus (56°C. for 1 hour) was used.

The results of a typical experiment with heated virus are also shown in Table II. It will be seen that 4 units of heated virus was inhibited by a much higher dilution of normal allantoic fluid than was an equal amount of unheated virus. In Table III the results of a number of experiments carried out with different

TABLE II
Inhibition Titer of Normal Allantoic Fluid with Infectious and Heated Virus

Infected allantoic fluid		Dilution of normal allantoic fluid*										Inhibition titer
Treatment	Hemagglutinating units per tube	2	4	8	16	32	64	128	256	512	1024	
None	4	0	0	0	1	2	3	4	4	4	4	8
Heated†	4	0	0	0	0	0	0	0	0	2	4	256

* Expressed as the reciprocal.

† 56°C. for 1 hour before dilution.

TABLE III
Relation between Hemagglutination Titer of Virus Pool and Inhibition Titer Obtained with Normal Allantoic Fluid

Infected allantoic fluid pool	Hemagglutination titer	Inhibition titer of normal allantoic fluid*
1	1024	32
2	2048	64
3	2048	128
4	4096	128
5	8192	128
6	8192	256

* Determined with 4 units of virus after heating.

pools of heated virus are summarized. In each instance the titer of inhibitor represents the highest dilution of normal allantoic fluid which inhibited 4 units of heated virus. Because the various virus pools had different titers, the dilution employed varied from one pool to another. It will be seen that 4 units of heated virus, when derived from a high titer pool, was inhibited by a considerably higher dilution of normal allantoic fluid than an equal number of units of heated virus derived from a pool of lower titer. It appears, therefore, that the titer of inhibitor in normal allantoic fluid is influenced by the hemagglutination titer of the virus suspension employed to determine it.

Centrifugation of Inhibitor.—The behavior of the inhibitor in normal allantoic

fluid towards influenza virus suggested that it might be a single component and of moderately large size. This impression was strengthened when it was found not to dialyze through cellophane membranes. To obtain information as to the size of the inhibitor relative to that of influenza virus, the effect of high gravitational fields on the inhibitor and on influenza virus was compared. Svedmyr (7) found that the inhibitor was sedimented at 27,000 R.P.M.

Nitrocellulose tubes containing 20 cc. of either normal allantoic fluid or allantoic fluid from embryos infected with PR8 were centrifuged at 15,000 R.P.M. for varying periods in a high speed vacuum apparatus (11). Both normal and infected fluids were centrifuged simultaneously. The centrifuge head was similar to that previously described (12) but was almost twice as large; the diameter = 30.1 cm. After centrifugation the top 10 cc. of each supernate was removed carefully, the remainder of the supernate was then withdrawn, and the sediment was resuspended in a quantity of buffered saline equal to the original volume. The inhibitor titer of the normal fluid fractions was determined as described above against 4 units of heated virus and the hemagglutination titer of the infected fluid fractions was determined in the usual manner.

The results are presented in Fig. 2 in which the upper portion (A) shows the titers obtained with the top 10 cc. of the supernates and the lower portion (B) shows those obtained with the resuspended sediments. Both the inhibitor and hemagglutination titers are plotted as logarithms. It will be noted that the titer of the inhibitor in the supernates decreased at a much slower rate than did that of the virus and, in addition, that with the resuspended sediments the virus titer increased at a faster rate than did the inhibitor titer. These results indicate that the inhibitor in normal allantoic fluid is significantly smaller than the virus particle (PR8) but show that some sedimentation of the inhibitor is obtained even at a speed of 15,000 R.P.M.

Heat Stability of the Inhibitor.—The effect of heat upon the inhibitor was determined in the following manner:

Specimens of normal allantoic fluid which had been dialyzed overnight against buffered saline (pH 7.2) were heated in a water bath at temperatures of 70 or 100°C. for varying periods. After this treatment the inhibitor concentration of each specimen was determined against 4 units of heated virus as described above. The allantoic fluid, which was almost water-clear before heating, developed only a very slight turbidity even when heated at 100°C. for 1 hour.

The results obtained are shown in Table IV. A twofold decrease in the inhibitor titer occurred after 45 minutes at 70°C. but no further drop in titer was found after 60 minutes. On heating at 100°C. the inhibitor titer decreased somewhat more rapidly but approximately 25 per cent of the inhibitor remained demonstrable even after 1 hour. It is evident that the inhibitor is very stable to heat. Svedmyr (7) showed previously that the inhibitor was not completely destroyed by similar heating.

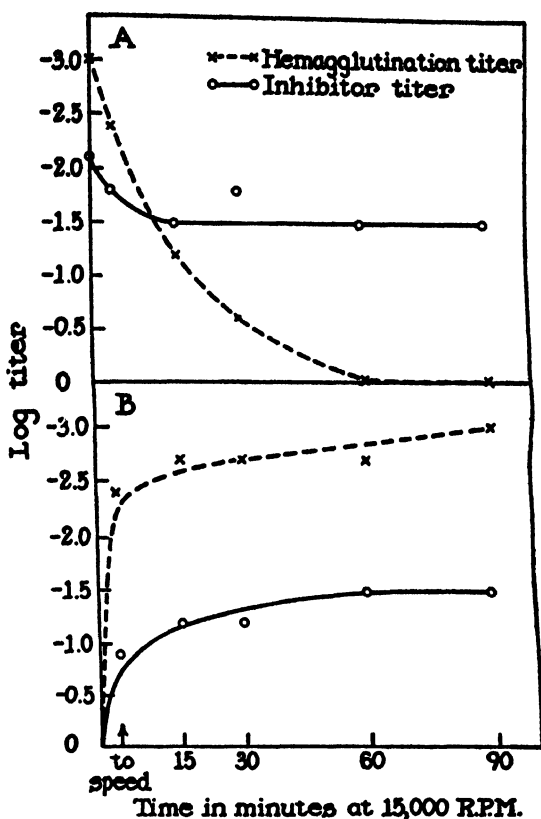


FIG. 2. Effect of high speed centrifugation on the inhibitor as compared with the virus. Aliquots of normal allantoic fluid and infected allantoic fluid were centrifuged simultaneously. Both the hemagglutination titer with infected fluid and the inhibitor titer with normal fluid of the top 10 cc. of the supernates, as shown in Graph A, and of the resuspended sediments, as shown in Graph B, are plotted against the time of centrifugation.

TABLE IV
Effect of Heating on Inhibitor

Treatment of normal allantoic fluid		Inhibition titer*	Treatment of normal allantoic fluid		Inhibition titer*
°C.	min.		°C.	min.	
—	—	128	—	—	128
70	15	128	100	15	128
70	30	128	100	30	64
70	45	64	100	45	64
70	60	64	100	60	32

* Determined against 4 hemagglutination units of heated virus.

Effect of pH on the Inhibitor.—The pH stability range of the inhibitor was determined in the following manner:

Normal allantoic fluid which had been dialyzed overnight against buffered saline was divided into aliquots. The pH of each aliquot was adjusted to the desired level by the dropwise addition of either 1 N HCl or 1 N NaOH. The specimens adjusted to pH 2.6 and 3.3 became slightly cloudy but no easily sedimentable precipitate formed; the fluid remained clear at the various other pH levels. After 1 hour at room temperature or 30 minutes at 70°C. the inhibitor titer of each specimen was determined against 4 units of heated virus.

The results are summarized in Table V. It will be seen that after 1 hour at room temperature the inhibitor was demonstrable in undiminished concentration at pH levels from 10.7 to 6.3, but at lower pH levels the titer decreased

TABLE V
Stability of Inhibitor at Different pH Levels

Normal allantoic fluid adjusted to†	Inhibition titer*	
	After 60 min. at room temperature	After 30 min. at 70°C.
pH		
2.6	16	2
3.3	16	2
4.6	32	16
5.3	32	32
6.3	64	64
7.0	64	64
9.8	64	32
10.0	64	32
10.7	64	16

* Determined against 4 hemagglutination units of heated virus.

† With 1 N HCl or 1 N NaOH.

progressively. After heating at 70°C. the inhibitor titer remained constant only at pH 7.0 and 6.3 and was reduced on both the alkaline and acid sides.

Effect of Temperature on Inhibition.—The effect of temperature on the inhibition of influenza virus (PR8) by normal allantoic fluid was studied by the constant inhibitor-decreasing virus technique described above. Identical titrations were carried out simultaneously at 4°C., room temperature (22–26°C.), and 37°C. with the same pools of virus.

The results of several such experiments are summarized in Table VI. The results of a similar experiment carried out with heated virus are also shown. It will be noted that the quantity of virus inhibited at 4°C. was from four- to eightfold greater than the quantity inhibited at room temperature; that the quantities of virus inhibited at room temperature and at 37°C. were almost identical; that when heated virus was employed there was very little difference

between the quantity inhibited at 4°C. and the quantity inhibited at room temperature; and that the quantity of heated virus inhibited at room temperature was the same as the quantity of infectious virus inhibited at 4°C. These results show that the inhibition of hemagglutination by normal allantoic fluid can be increased by lowering the temperature to 4°C., and that the same increase in inhibition can be obtained at room temperature or 4°C. by the use of heated virus.

Action of Enzymes and Periodate on the Inhibitor.—Evidence as to the nature of the inhibitor was sought by studying the effect of different purified enzymes upon its capacity to inhibit hemagglutination. It was found that crystalline trypsin¹ in low concentration failed to alter the inhibitor titer. However, when allantoic fluid which had been dialyzed against buffered saline (pH 7.2)

TABLE VI
Effect of Temperature on Quantity of Virus Inhibited by a Constant Amount of Normal Allantoic Fluid

Infected allantoic fluid pool	Hemagglutination titer*			
	In buffer saline	In normal allantoic fluid		
		4°C.	22-26°C.	37°C.
A	256	32	128	256
B	1024	32	128	128
C	2048	16	128	128
B heated†	1024	16	32	—

* Expressed as the reciprocal.

† 56°C. for 1 hour.

was treated with a relatively high concentration of crystalline trypsin (1.25 mg. per cc.) for 3 hours at 37°C., there was almost complete destruction of inhibitor activity as is shown in Table VII. It is well established that egg white contains a trypsin inhibitor in very high concentration (13), and it seems probable that a similar substance may be present in allantoic fluid. If this were true, it would provide an explanation for the failure of trypsin in low concentration to destroy inhibitor activity.

The effect of crystalline ribonuclease and highly purified desoxyribonuclease on the inhibitor was also determined.

An equal volume of crystalline ribonuclease² (1.0 mg. per cc.) or desoxyribonuclease² (1.0 mg. per cc. in 0.03 M MgSO₄) was added to dialyzed allantoic fluid and the mixtures were

¹ Obtained through the courtesy of Dr. M. Kunitz of The Rockefeller Institute, Princeton, New Jersey.

² These enzymes were kindly provided by Dr. M. McCarty of the Hospital of The Rockefeller Institute, New York.

incubated at 37°C. for 4 hours. Following this the inhibitor titer of each mixture was determined against 4 units of heated PR8.

The results obtained are also shown in Table VII. It will be noted that neither enzyme had any apparent effect upon the inhibitor titer.

Burnet (8) showed that several mucinous substances inhibit hemagglutination by influenza viruses. This prompted the treatment of allantoic fluid with several hyaluronidase preparations.

Four preparations were used: a pneumococcal hyaluronidase³ (containing 1500 viscosity units per mg.) prepared from a type II R (D39R) culture by the method of Meyer *et al.* (14);

TABLE VII
Effect of Enzymes and Periodate on Inhibitor

Treatment of normal allantoic fluid			Inhibitor titer	
Material	Concentration	Time	After treatment	Control not treated
	mg./cc.	hrs.		
Pneumococcal hyaluronidase.....	0.001	1	<2	256
Pneumococcal hyaluronidase (heated)*.....	0.001	1	256	256
Crystalline trypsin.....	1.25	3	4	128
Crystalline ribonuclease.....	0.5	4	128	128
Desoxyribonuclease†.....	0.5	4	128	128
	per cent			
Streptococcal extract.....	10.0	1	64	64
Leech extract.....	10.0	1	64	64
Testicular extract.....	10.0	1	64	64
LiIO ₄	0.025 M	2	<4	64

* 70°C. for 5 minutes in buffered saline.

† In 0.03 M MgSO₄.

a streptococcal extract³ (approximately 100 viscosity units per cc.) prepared from the supernate of a culture of group A, type 4 hemolytic streptococci; a crude bull testicular extract³ (570 viscosity units per cc.) prepared by the method of Kass and Seastone (15); and a leech extract³ (approximately 500 viscosity units per cc.) prepared by the method of Claude (16). The pneumococcal hyaluronidase was dissolved in distilled water and added to dialyzed normal allantoic fluid to yield a concentration of 0.001 mg. per cc.; the streptococcal, testicular, and leech extracts were added to specimens of allantoic fluid to yield a concentration of 10 per cent. The fluids were incubated in a water bath at 37°C. for 1 hour and then at 70°C. for 5 minutes in order to inactivate the various enzymes. After treatment the inhibitor titer of each mixture was determined against 4 units of heated PR8.

The results of these experiments are presented in Table VII. It will be seen that there was no demonstrable inhibitor in allantoic fluid after treatment

³ Kindly provided by Dr. S. Rothbard of the Hospital of The Rockefeller Institute, New York.

with pneumococcal hyaluronidase, whereas the inhibitor titer was undiminished in the fluids treated with streptococcal, testicular, or leech extracts. This raised the possibility that the component of the pneumococcal preparation responsible for the destruction of the inhibitor might be something other than hyaluronidase. The preparation did contain a hemolysin but had no proteolytic activity against gelatin or casein.⁴ The ability of the pneumococcal enzyme to destroy the inhibitor was completely inactivated after heating for 5 minutes at 70°C. in the presence of allantoic fluid, buffered saline, or phosphate buffer (pH 7.1, μ 0.5); the hyaluronidase activity as determined by the viscosimetric method was also destroyed under these conditions.⁴

Meyer *et al.* (17, 18) compared hyaluronidases of different origin by both viscosimetric and reductometric methods and found that pneumococcal hyaluronidase hydrolyzed the substrate to almost 100 per cent of the theoretical amount, but that testicular and leech hyaluronidases, both of which contained more activity than the pneumococcal preparations in viscosimetric tests, hydrolyzed the substrate to only 50 and 40 per cent, respectively. They concluded that hyaluronidases were mixtures of at least two enzymes, one attacking the long chain molecules, the other hydrolyzing the aldobionic acid units. This could provide an explanation for the failure of the streptococcal, testicular, and leech extracts to destroy inhibitor activity. The very low concentration of pneumococcal hyaluronidase required to destroy inhibitor activity supports the idea that one of the hyaluronidase enzymes was responsible for the destruction of inhibitor activity.

Hirst (10) showed that sodium periodate destroys the virus receptors of red blood cells and also the hemagglutination inhibitor in normal rabbit serum. He suggested that this effect was due to the alteration of a polysaccharide by periodate. The effect of periodate on the inhibitor in allantoic fluid was therefore studied.

One volume of 0.1 M LiIO_4 was added to 3 volumes of normal allantoic fluid. After 2 hours at room temperature 2 volumes of 7.5 per cent glucose was added to inactivate the remaining periodate, and the inhibitor titer was then determined against 4 units of heated PR8.

The results of this experiment are shown in Table VII. It will be seen that insofar as could be determined the inhibitor was completely inactivated by lithium periodate.

It appears that three substances have been found, each of which is capable of inactivating the inhibitor in normal allantoic fluid. These are: crystalline trypsin, pneumococcal hyaluronidase, and lithium periodate. The destruction of inhibitor activity by crystalline trypsin suggests that at least part of the inhibitor is protein, while inactivation by pneumococcal hyaluronidase as well as periodate suggests that the inhibitor contains carbohydrate. The available

⁴ These determinations were kindly carried out by Dr. M. McCarty of the Hospital of The Rockefeller Institute, New York.

evidence is consistent with the idea that the inhibitor is, in all probability, a mucoprotein.

Combination between Inhibitor and Virus.—It seemed possible that the inhibitor might prevent hemagglutination in either one of two ways: it could block the virus receptors on the RBC, or it could combine with the virus itself. In order to test the first possibility, chicken RBC were suspended in normal allantoic fluid for varying periods, then sedimented and resuspended in buffered saline. Such treated RBC were found to be agglutinated by both infectious and heated virus equally as well as untreated RBC. These results indicate that the inhibitor did not combine with virus receptors on the RBC. In order to determine whether the inhibitor combined with the virus itself, the following experiment was carried out.

Serial twofold dilutions of normal allantoic fluid were made in duplicate in buffered saline. To each dilution were added 4 units of infectious PR8 and sufficient RBC to yield a concentration of 0.25 per cent. To similar duplicate dilutions of normal allantoic fluid were added 4 units of heated virus and 0.25 per cent RBC. The inhibitor titer with one series of dilutions containing infectious virus and one containing heated virus was determined in the usual manner. In the case of the other two series the RBC were sedimented immediately after mixing by centrifugation at 2,500 R.P.M. for 2 minutes, the supernate from each tube was discarded, and the cells were resuspended in a quantity of buffered saline equal to the original volume. In every instance the inhibitor titer was determined after 1 hour at room temperature.

The results obtained from this experiment are shown in Table VIII. It will be noted that the inhibitor titer determined against 4 units of infectious virus in the usual manner was very low but that with the duplicate titration, in which the RBC were sedimented immediately and resuspended in saline, the apparent inhibitor titer was 16-fold higher. This indicates that the virus was not sedimented with the RBC in those tubes which contained sufficient inhibitor. When heated virus was used, the inhibitor titer was high in the case of both titrations. The results of this experiment suggest that the inhibitor combines with the virus itself and thereby prevents adsorption of the virus by RBC; that infectious virus is eluted rapidly although incompletely from the inhibitor and is then capable of uniting with RBC; and that heated virus is almost completely incapable of dissociating from the inhibitor just as it is incapable of dissociating from RBC (19). It seems apparent that once the virus-inhibitor reaction has reached equilibrium RBC are incapable of causing the virus to dissociate from inhibitor. If this were not the case, inhibition of hemagglutination in the presence of normal allantoic fluid would not be demonstrable.

The Rate of the Reaction between Virus and Inhibitor.—It was suggested by the results obtained in the preceding experiment that influenza virus combined with inhibitor and then rapidly although incompletely dissociated from it. In order to obtain additional information concerning the reaction between virus and inhibitor, the following experiment was carried out.

Serial twofold dilutions from 1:2 to 1:16 of virus were made in undiluted normal allantoic fluid and immediately placed in a water bath at 37°C. Aliquots of each dilution mixture were removed after varying times and immediately heated at 56°C. for 1 hour in order to stop the reaction between virus and inhibitor. The hemagglutination titer of each aliquot was then determined in the usual manner. The inhibitor titer of each aliquot against 4 units of heated virus was also determined after the aliquot had been heated additionally at 70°C. for 5 minutes in order to completely eliminate the hemagglutinating activity of the virus originally present in it.

The results of this experiment are presented graphically in Fig. 3 in which the upper portion (A) shows the virus hemagglutination titers and the lower

TABLE VIII
Evidence for Combination between Inhibitor and Virus

Mixture				Centrifugation*	Dilution of normal allantoic fluid													Inhibitor titer
PR8-infected allantoic fluid		Normal allantoic fluid	RBC															
Treatment	Hemagglutinating units per tube				2	4	8	16	32	64	128	256	512	1024	2048			
None	4	Indicated dilution	0.25	None	0	0	0	1	2	3	4	4	4	4	4	8		
	4	Indicated dilution	0.25	2500	0	0	0	0	0	0	0	1	4	4	4	128		
Heated†	4	Indicated dilution	0.25	None	0	0	0	0	0	0	0	0	2	4	4	256		
	4	Indicated dilution	0.25	2500	0	0	0	0	0	0	0	0	0	2	4	512		

* Mixture centrifuged immediately after preparation. Sedimented RBC resuspended to volume in buffered saline.

† Undiluted fluid heated at 56°C. for 1 hour.

portion (B) the inhibitor titers plotted as logarithms against the time of incubation at 37°C. It will be noted that, when the mixtures of virus in normal allantoic fluid were heated at 56°C. for 1 hour immediately after preparation, hemagglutination was demonstrable only in the mixture with the lowest dilution of virus, *i.e.* 1:2, and then only in very low titer. However, when the mixtures were incubated at 37°C. for varying times before heating at 56°C., the hemagglutination titer of each mixture increased progressively with time. The rate of increase in titer became slower as the ratio of virus to normal allantoic fluid decreased, and the maximum titer reached with each mixture was progressively lower than would have been expected in terms of the quantity of virus present in the mixture. The inhibitor titers determined with the same

mixtures showed that the quantity of demonstrable inhibitor decreased progressively as the hemagglutination titer increased, and the inhibitor completely disappeared from each mixture at the same time that the hemagglutination titer reached a maximum level except with the mixture which originally con-

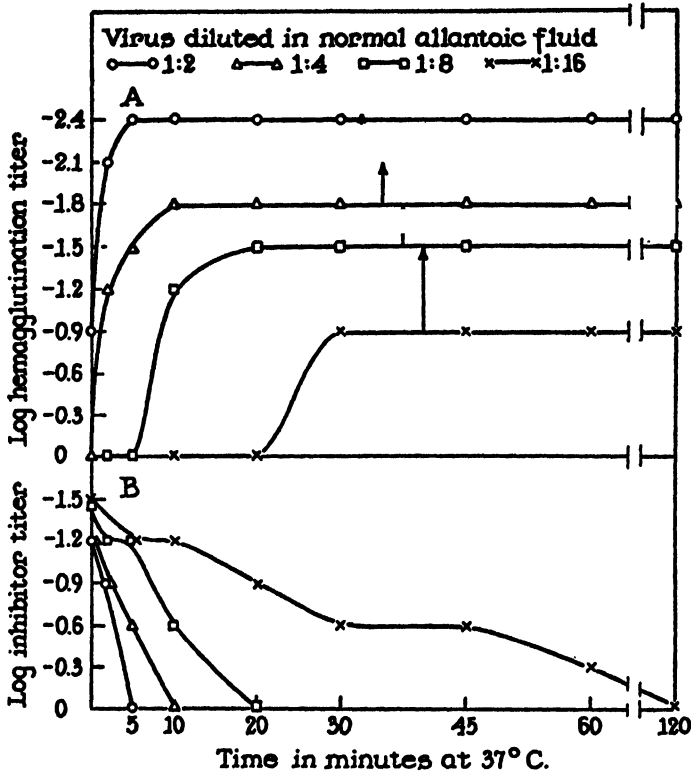


FIG. 3. The rate of the reaction between virus and inhibitor. Serial twofold dilutions of virus in allantoic fluid were incubated at 37°C. Aliquots were removed after varying periods and immediately heated at 56°C. for 1 hour. The hemagglutination titer of each aliquot is shown in Graph A. The arrows above each curve indicate the level which the hemagglutination titer should have reached if all virus had been released from combination with inhibitor. The inhibitor titer against 4 units of heated virus, after each aliquot had been heated additionally at 70°C. for 5 minutes, is shown in Graph B.

tained a 1:16 dilution of virus. In this mixture there was still a small amount of demonstrable inhibitor present when the hemagglutination titer reached a maximum level, but even in this instance the inhibitor titer decreased on further incubation. These findings provide strong evidence that the virus combines with the inhibitor and then dissociates partially from it. They also indicate that combination between the virus and the inhibitor takes place very rapidly,

and that upon completion of the reaction the inhibitor is no longer demonstrable. Furthermore, the results of this experiment indicate that the quantity of virus which is capable of dissociating from inhibitor is dependent upon the ratio of virus to inhibitor; the lower the ratio, the less virus is released.

Quantitative Relation between Free and Combined Virus.—Additional information concerning the effect of the virus-inhibitor ratio on the quantity of virus capable of remaining combined with inhibitor was obtained in the following manner:

Serial twofold dilutions of infectious virus were made in buffered saline and to each dilution was added an equal quantity of normal allantoic fluid. Thus, the inhibitor concentration in the resulting mixtures remained constant while the virus concentration progressively decreased. The mixtures were held at room temperature for 30 minutes or longer so as to reach equilibrium. In certain experiments the mixtures were held at 37°C. for as long as 24 hours. The hemagglutination titer of each mixture then was determined in the usual manner. The quantity of virus combined with inhibitor, *i.e.* the quantity of virus incapable of dissociating from inhibitor, in each mixture was taken as the difference between the total quantity of virus added to the mixture and the quantity of free virus demonstrable by hemagglutination.

The results obtained in three separate experiments are plotted graphically in Fig. 4: curve I was fitted to points which show the logarithm of the quantity of virus combined with inhibitor plotted against the logarithm of the total quantity of virus added; curve II was fitted to points which show the logarithm of the quantity of free virus plotted against the logarithm of the total quantity of virus added. It will be noted that as the total amount of virus in the mixtures decreased, *i.e.* as the ratio of virus to inhibitor decreased, relatively less free virus was demonstrable, and a higher proportion of the total virus remained combined with inhibitor. Under these circumstances it would be expected that curves I and II should diverge from each other as, it will be seen, they do. Thus it appears that in the presence of a constant concentration of inhibitor the quantity of virus which remains combined at equilibrium is a function of the total quantity of virus present in the mixture. In this connection it is of importance to recall that with infected allantoic fluids which showed various hemagglutination titer levels, *i.e.* free virus, the quantity of virus which combined with a constant amount of added inhibitor was directly related to the free virus level (*cf.* Table II). It appears that the reaction between influenza virus and inhibitor can be expressed in terms of the usual equilibrium equation:



in which VI represents virus (V) combined with inhibitor (I). It should, however, be emphasized that the inhibitor is altered by the virus as is the case also with the inhibitor present in normal serum (19). It was found that incubation of mixtures of virus and inhibitor at 37°C. for periods as long as 24 hours did not lead to any consistent alteration in the equilibrium; no additional

free virus was released from combination after the first few minutes of the reaction.

Inhibitor in Egg White.—Numerous attempts were made to concentrate and purify the inhibitor in normal allantoic fluid by a number of procedures with

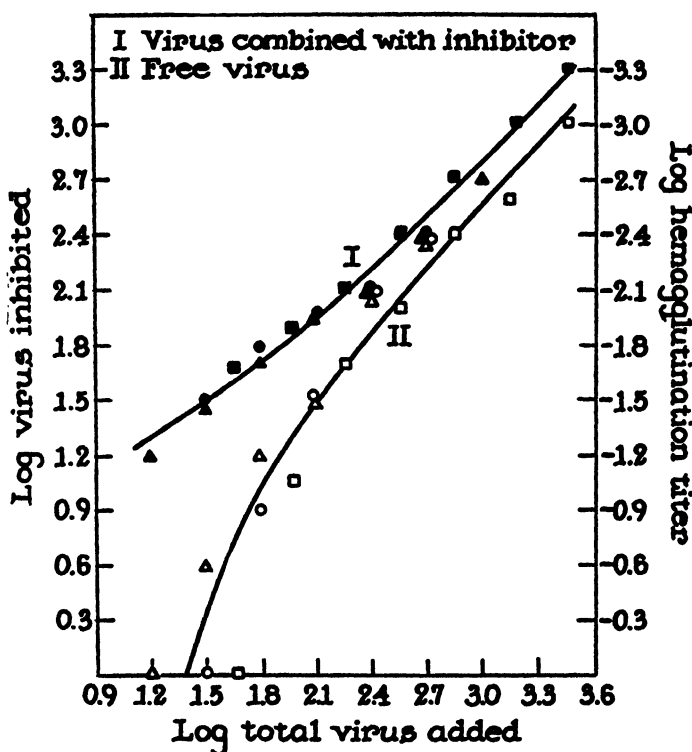


FIG. 4. The equilibrium between virus and inhibitor. Equal portions of undiluted allantoic fluid were added to serial twofold dilutions of virus. After 30 minutes at room temperature the quantity of free virus in each mixture was determined by the hemagglutination technique. The quantity of virus combined with inhibitor, curve I, was determined from the difference between the total quantity of virus added and the quantity of free virus found, curve II.

very little success. This was partially attributable to the very low concentration of the inhibitor and therefore a richer source derived from the egg was sought. It was found that egg white in very high dilution was capable of inhibiting hemagglutination: a 0.25 per cent solution of egg white in saline had approximately the same inhibitor activity as undiluted normal allantoic fluid. The inhibitor in egg white was found to react with influenza virus in the same manner as does that in allantoic fluid, and its properties, *i.e.* heat stability, pH stability, sedimentation in the centrifuge, and inactivation by enzymes, were

closely similar to those of the inhibitor in allantoic fluid. Therefore, it appeared that the two substances were very closely related if not identical.

Purification of the inhibitor present in egg white was undertaken.

Ovalbumin, ovomucoid A (ovomucin), and lysozyme, substances fairly readily separated from egg white, were found to have little or no inhibitor activity. However, it was found that most of the inhibitor could be sedimented with the precipitate which formed when egg white was diluted with 3 to 4 volumes of distilled water, or when a solution of egg white in 1 per cent saline was brought to 0.3 saturation with ammonium sulfate. Slight purification could be obtained with either method by redissolving the precipitate in 1 per cent saline adjusted to pH 8.0 and reprecipitating several times as before. However, with each additional precipitation the material became increasingly more insoluble.

The most highly purified preparation of the inhibitor was obtained when egg white was fractionated by a cold-alcohol precipitation method adapted from that devised by Cohn *et al.* (20) for fractionating serum proteins. By this method most of the inhibitor activity was found in the fraction which was precipitated at -3°C ., pH 7.9, and an ethanol concentration of 25 per cent. This fraction was extremely difficult to put into solution from the dried state even with violent stirring. It was found that the final material in a concentration of about 0.06 mg. per cc. in 1 per cent saline inhibited the same quantity of heated virus as does normal allantoic fluid. On this basis it can be computed that 1 unit of heated virus should be inhibited by approximately 0.5 μg . of the purified material. Chemical analyses on this fraction were not made but it was found to give both a positive Molisch reaction for carbohydrate and a positive biuret reaction for protein.

Inhibitor in Infected Allantoic Fluid.—On numerous occasions attempts were made to demonstrate the presence of inhibitor in infected allantoic fluid after the virus had been either inactivated or removed. The virus was inactivated by either heat or treatment with alkaline pH; *i.e.*, 10 or more; it was removed from the allantoic fluid by either centrifugation or cold-alcohol precipitation. On no occasion was it possible to demonstrate any inhibitor activity whatsoever in infected fluid. In view of the results of the various experiments described above, it would not be expected that inhibitor could be demonstrated in infected allantoic fluid. Because of the prolonged opportunity for contact with virus during the course of multiplication, it would be anticipated that some of the inhibitor should be combined with virus while the remainder, although dissociated from combination with virus, should be altered as a result of viral action. In neither instance could present methods demonstrate the presence of the component.

Bound Virus in Infected Allantoic Fluid.—It is evident from the results of preceding experiments that virus bound to inhibitor is unable to agglutinate RBC. There is, however, no evidence that bound virus is incapable of adsorption to RBC. Nonetheless, it seemed possible that, if part of the virus in

infected allantoic fluid actually were bound to inhibitor, the free virus in the fluid might be separated from the bound virus by adsorption onto and elution from RBC. If such a separation were accomplished, then free virus alone should react differently with added fresh inhibitor than the original infected fluid, *i.e.* more free virus should be bound by fresh inhibitor in the absence of bound virus than in its presence because, as is shown above, the total quantity of virus which combines with inhibitor is directly related to the ratio of virus to inhibitor. However, it was found that virus which had been adsorbed onto and eluted from RBC reacted with fresh inhibitor in a manner quantitatively identical to that of the original infected fluid. It would appear, therefore, that adsorption onto and elution from RBC does not provide evidence of a separation of free from bound virus.

It was found also that there was no significant reduction in the embryo infectivity titer of virus which had been serially diluted in normal allantoic fluid so as to provide a great excess of inhibitor; the 50 per cent embryo infectivity titer was $10^{-8.0}$, whereas that of the control was $10^{-8.5}$. This indicates that virus bound to inhibitor is equally as infectious for the embryo as is free virus. It has been shown (21) that when influenza virus is adsorbed onto RBC from infected allantoic fluid, the extent of the decrease in the hemagglutination titer is closely paralleled by a decrease in the infectivity titer. This evidence suggests that bound virus is adsorbed onto RBC to an extent similar to that of free virus. If this were not the case, the hemagglutination titer, which is a measure of free virus, should be decreased by RBC adsorption to a greater extent than the infectivity titer.

DISCUSSION

It appears evident from the results obtained in this study that there is in normal allantoic fluid a component which is capable of combining with influenza virus and that virus which is combined with the component is incapable of causing hemagglutination. When normal allantoic fluid is added to infected allantoic fluid, the inhibiting component present in the former fluid promptly combines with the free virus present in the latter fluid. This reaction reaches equilibrium in a short period of time and the relative proportions of the components which enter into it then remain constant so long as their concentrations are not changed by the addition of more inhibitor or more virus. Present evidence indicates clearly that although some of the virus dissociates from the inhibiting component after combination not all of the virus is released from combination and a relatively large proportion remains in stable union with the component. As a result of experiments in which the concentrations of virus and inhibitor were varied with respect to each other, it was found that the quantity of virus which remains combined with a constant amount of inhibitor and obviously also the quantity which dissociates from inhibitor are

directly related although not strictly proportional to the quantity of virus present in the system.

It is apparent that there is a striking difference between the virus-inhibitor reaction and the virus-erythrocyte reaction. The latter reaction appears to proceed to completion and all or almost all the virus dissociates from RBC (21). The former reaction appears not to go to completion but instead reaches a fixed equilibrium. Over the range of variables studied the smallest proportion of virus which remained combined with inhibitor was at least 50 per cent and the largest 100 per cent. Following partial dissociation of the virus-inhibitor complexes, the inhibitor which is released is so altered that it can no longer be demonstrated directly by available techniques. In this respect, the reaction is analogous to the virus-erythrocyte reaction. It is well established that on dissociation of the latter combination the RBC also are altered and do not again combine with the virus (21). It follows directly from these considerations that whatever quantity of the inhibiting component may be free in infected allantoic fluid would be expected to be similarly altered and not demonstrable as a consequence. It will be recalled that all attempts to demonstrate the presence of unaltered inhibitor in infected fluid were unsuccessful. It is, of course, possible that infected fluid is actually devoid of the inhibiting component and that all which was present at the time infection was initiated was not only altered as a result of contact with the virus but actually was destroyed or otherwise removed from the allantoic fluid during the course of infection. This seems a forced and unlikely assumption, particularly because there is evidence which indicates that virus combined with inhibitor is present in infected fluid. In the light of the quantitative relationship between inhibitor concentration and virus concentration it can be shown that, if inhibitor remains present and in constant concentration in infected allantoic fluid, proportionately more virus should be combined in fluids of low virus concentration than in fluids of high virus concentration. Therefore, the addition of fresh inhibitor to infected fluids should lead to the binding, *i.e.* inhibition, of less free virus in fluids of low virus concentration than in fluids of high virus concentration. It was found that such a relationship can be demonstrated.

The evidence obtained in this study makes it appear highly probable that allantoic fluid infected with influenza virus contains at least three components which are in equilibrium: (1) free virus (which is capable of causing hemagglutination), (2) altered inhibitor (which is no longer capable of combining with virus), and (3) virus combined with inhibitor. Under these circumstances the addition of normal allantoic fluid to infected allantoic fluid serves merely to upset an existing equilibrium which then promptly becomes reestablished at a different level. The increased inhibitor concentration of the system should lead to an increase in the amount of bound virus at the expense of free virus. The evidence indicates that this occurs. It is important to point out that virus

combined with inhibitor appears to be equally as infectious for the chick embryo as free virus.

Many of the characteristics of the hemagglutination reaction with influenza virus suggest that erythrocytes are agglutinated as a direct result of their combination with virus particles and not because of secondary alterations produced in the red blood cells. Indeed, Heinmets (22) in studies on the RBC-virus reaction with the electron microscope obtained evidence which suggests that virus particles form linkages between erythrocytes and thereby cause them to agglutinate. There appear to be adequate reasons for thinking that the virus is "divalent" or possibly "multivalent" with respect to red blood cells; *i.e.*, a single virus particle can combine with more than one erythrocyte simultaneously. If it is assumed for purposes of simplification that the virus is merely divalent, *i.e.* can combine with but two erythrocytes simultaneously, then it is probable that both "valences" should be free or uncombined if hemagglutination is to occur. In terms of this hypothesis, if one valence were combined with inhibitor while the other remained free, hemagglutination should not occur even though the virus should still be capable of adsorption onto and elution from RBC. It seems likely that such a situation may actually exist in infected allantoic fluid and that some of the virus which is combined with inhibitor can also unite with erythrocytes. That RBC can adsorb virus particles without becoming agglutinated has been shown recently (22). In view of these considerations it would appear that present procedures for the purification of influenza viruses probably do not permit of a complete separation of virus particles from combined inhibitor. In this connection, it is of interest that Cohen (23) found that highly purified influenza virus preparations obtained from infected allantoic fluid contained a considerable amount of antigenic material characteristic of the host. Knight (24) obtained similar results with highly purified preparations from both allantoic fluid and mouse lung suspensions and expressed the opinion that host material was incorporated into the virus particle itself. The results of the present study suggest that the host component, *i.e.* inhibitor, is bound to the surface of the virus particle as a result of a definite reaction and is, in all probability, not an essential constituent of the virus.

On the basis of chemical analyses Knight (25) concluded that the virus particles contain a polysaccharide in addition to other substances. He also found an appreciable amount of glucosamine in sedimentable material from normal allantoic fluid and suggested that the amount of host material in purified virus-containing particles could be ascertained from their glucosamine content. Inasmuch as the results obtained in the present study indicate that the inhibitor present in allantoic fluid is probably a mucoprotein which is capable of forming a stable combination with the virus, it would appear that a simple

explanation may be offered for some of the results previously obtained in immunological and chemical studies on purified preparations.

The presence of virus-inhibitor complexes in infected allantoic fluid may have consequences of both practical and theoretical significance. Mention has been made of the difficulties of separating free virus from bound virus either in the centrifuge or by adsorption and elution from RBC. Because hemagglutination titrations serve to measure free virus, but not virus bound by inhibitor, they may not reflect accurately the total concentration of virus in an infected allantoic fluid. Moreover, if different strains of virus react in quantitatively different manners with inhibitor, an inconstant ratio between infectivity and hemagglutination titers might be anticipated. It is well known that freshly recovered strains may give very peculiar and irregular results both in hemagglutination and in hemagglutination-inhibition titrations in the presence of immune serum. It seems probable that certain of the unusual reactions obtained with such strains may be attributable to the presence of virus-inhibitor complexes.

SUMMARY

Evidence is presented which shows that there is a component present in normal allantoic fluid, probably mucoprotein in nature, capable of combining with influenza A virus (PR8), and that following combination between this component and the virus only partial dissociation of the complex occurs. Evidence is also presented which strongly suggests that the component is present in virus-infected allantoic fluid in which it is in part combined with the virus and in part free although altered by viral action. The probability that the component is present as well in highly purified preparations of influenza virus, and its effect upon various reactions obtained with this agent are discussed.

Addendum.—In a recent paper Lanni and Beard⁵ reported that egg white is highly effective in inhibiting hemagglutination by heated swine influenza virus and suggested that this capacity is attributable to a component which combines with the virus.

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QUANTITATIVE ANTISTREPTOKINASE STUDIES IN PATIENTS
INFECTED WITH GROUP A HEMOLYTIC STREPTOCOCCI: A
COMPARISON WITH SERUM ANTISTREPTOLYSIN AND
GAMMA GLOBIN LEVELS WITH SPECIAL REFERENCE
TO THE OCCURRENCE OF RHEUMATIC FEVER*

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The phenomenon of liquefaction of human fibrin clots by broth cultures or culture filtrates of *beta* hemolytic streptococci was first described by Tillett and Garner in 1933 (1). The complex mechanism of this reaction, thought at first to be a direct fibrinolytic effect of a streptococcal enzyme, has been elucidated in recent years. Milstone (2) demonstrated that a substance present in plasma, termed "lytic factor," is required for dissolution of the clot; and as a result of the work of Christensen (3, 4), confirmed independently by Kaplan (5), it is now known that the "lytic factor" is a proteolytic enzyme normally present in the plasma as an inactive precursor. The rôle of the active streptococcal substance is that of an activator of the proteinase precursor, converting it into an active enzyme in a manner analogous to the conversion of trypsinogen to trypsin by enterokinase. The active serum proteinase is responsible for digestion of the fibrin clot. In view of the accumulated evidence regarding the nature of streptococcal fibrinolysis, Christensen and MacLeod (6) have proposed the term *streptokinase* to replace the term fibrinolysin originally applied to the streptococcal component of the system. They have further suggested the name *plasminogen* for the inactive form of the serum proteinase and *plasmin* for the active enzyme. This terminology has been adopted in the present report.

The activity of streptokinase derived from group A hemolytic streptococci in promoting the lysis of human fibrin clots is in sharp contrast to its minimal effect on clots of other animal species (1, 4, 7, 8). Although the basis for the apparent human specificity remains somewhat uncertain, this property of streptokinase is unique and other products of streptococcal cells, such as streptolysin, erythrogenic toxin, and streptococcal proteinase do not exhibit comparable specificity. Thus, streptokinase deserves special attention in the study of rheumatic fever, which is a sequela of streptococcal infection that appears to be limited to the human species.

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Numerous immunological studies on streptokinase (fibrinolysin) have been carried out with human plasma (9, 10, 11, 12, 13) using the antifibrinolysin test described by Tillett and Garner (1), but because of the essentially qualitative nature of the test, it has not been possible to obtain definitive information comparable to that derived from the more precise quantitative titrations of other serum antibodies. Kaplan, in collaboration with the Commission on Acute Respiratory Diseases, published in 1946 (14) the first quantitative method for estimation of antibody directed against streptokinase. This method involves a neutralization test in which a constant, standardized amount of streptokinase is incubated with serial dilutions of the serum to be tested, following which an indicator system consisting of fibrinogen, plasminogen and thrombin is added. The end-point of the test is defined as the reciprocal of the highest dilution of serum which completely prevents lysis of the clot during a second period of incubation. The results of individual titrations proved to be sufficiently reproducible on repeated tests to establish the method as a useful quantitative procedure. The most serious objection that can be raised to the method is that it has not been possible up to the present time to standardize the plasminogen-streptokinase system in specific units so that antibody titers obtained in one laboratory can be compared directly with those of other laboratories. In a given study, however, valuable data can be obtained by using a single lot of streptokinase and of human plasma Cohn Fraction I (containing both fibrinogen and plasminogen) for the entire series of tests.

In the present study, quantitative antistreptokinase determinations have been carried out on serial specimens of serum obtained from patients during an epidemic of scarlet fever. The primary aim of the investigation was to determine whether or not significant differences in the pattern of antistreptokinase response exist between patients who developed rheumatic fever subsequent to the attack of scarlet fever and those who did not. Antistreptolysin O titers and the gamma globulin levels of the same sera are presented for comparison.

Materials and Methods

Buffered saline solution: 0.05 M veronal buffer, pH 7.5, diluted to 0.01M with 0.85 per cent NaCl solution was used throughout in diluting the various reagents. This solution is hereafter referred to as buffered saline solution.

Fibrinogen and plasminogen: A lyophilized preparation of Fraction I (Cohn) of human plasma¹ served as the source of both fibrinogen and plasminogen. A 0.6 per cent solution in buffered saline was used both in standardizing the streptokinase preparation and in the quantitative antibody determinations. Solutions of this concentration formed a firm clot promptly upon the addition of thrombin.

Thrombin: Topical thrombin,² of bovine origin, supplied in ampoules containing

¹ We are indebted to Sharp and Dohme, Inc., for a generous supply of Fraction I.

² Manufactured by Parke, Davis and Co.

5000 units, was dissolved in 5 cc. of physiological saline solution and further diluted 1:10 in buffered saline solution.

Streptokinase: A single lot of streptokinase was used throughout these experiments. A strain of group A hemolytic streptococcus, H105,^{*} was grown overnight in neopeptone dialysate broth (15). The next morning sterile 50 per cent glucose solution (40 cc. per liter of culture) and phenol red (10 mg. per liter of culture) were added to the culture and incubation continued another five hours during which time the pH of the culture was maintained at or slightly above 7.0 by the frequent addition of 5N NaOH. This procedure resulted in a tenfold increase of streptokinase activity. The culture was centrifuged and the supernate filtered through a Coors No. 3 filter. Approximately 300 cc. of nondialyzed neopeptone broth had previously been passed through this filter, because it was found that a filter not so treated quantitatively absorbs streptokinase out of dialyzed neopeptone broth. One-cc. amounts of the sterile filtrate were placed in small tubes and stored in a CO₂ chest at -70° C.

Standardization of streptokinase: The procedures used for the standardization of streptokinase and for the quantitative determination of antistreptokinase are similar to, but not identical with, those described by Kaplan (14). In each of a series of tubes were placed the following: 1.0 cc. of varying dilutions of streptokinase, 1.0 cc. of 0.6 per cent Fraction I solution, and 0.2 cc. of the thrombin solution. The tubes were immediately shaken and, when clotting had occurred, were incubated in a water bath at 37° C. for 30 minutes. The highest dilution of streptokinase which just effected complete dissolution of the fibrin clot was used in the determination of anti-streptokinase.

Determination of antistreptokinase in serum: Twofold dilutions of serum were used, beginning with a dilution of 1:10. To 0.5 cc. of the serum dilutions was added 0.5 cc. of streptokinase in the dilution determined by the method outlined above. These tubes were mixed by shaking and then incubated in a water bath at 37° C. for 30 minutes to allow for antigen-antibody combination. According to Kaplan (16) this is 95-98 per cent complete in 30 minutes. The tubes were then placed in an ice-water bath to prevent as far as possible any enzymatic activity during the time required for the addition of the indicator system. To each tube was added 1.0 cc. of the 0.6 per cent Fraction I solution (containing plasminogen and fibrinogen). There was then added 0.2 cc. of the thrombin solution and the tubes shaken immediately to insure the formation of a uniform clot. The tubes were re-incubated at 37° C. for 60 minutes. The criteria for reading the test are those described by Kaplan. The end-point was taken as the reciprocal of the serum dilution which completely prevented lysis of the clot as determined by the failure of the clot to slide when the tube was gently tapped in the inverted position. As a control on the activity of the streptokinase preparation, a tube in which the serum dilution was replaced by buffered saline solution was included in each series of dilutions. Complete lysis was expected in this tube. The largest number of sera which could be conveniently tested at one time was found to be six. An indication of the reproducibility of the test is given by the results of 50 repeat titrations. The end-points were identical in 38, and varied

^{*} Strain H105 was obtained from Dr. W. S. Tillett and was designated Co in his laboratory (1).

by only one tube in the remaining 12. Therefore, a rise in antibody titer of two or more tubes was considered to be significant.

Antistreptolysin O: The method used for the determination of antistreptolysin O was that described by Todd as modified by Hodge and Swift (17). A two-tube rise in titer was considered significant. It should be noted that the dilution increments in the determination of antistreptolysin O differ from those used in the antistreptokinase test.

Gamma globulin: The gamma globulin levels were determined by the turbidimetric method described by Kunkel (18). The turbidity readings were converted into values expressed in gms. per cent on the basis of standardization of the turbidimetric readings by electrophoretic determination of gamma globulin.

Titration of the streptokinase produced by individual strains of group A hemolytic streptococci: The procedure used is similar to that reported by the Commission on Acute Respiratory Diseases (19). A lyophilized culture of the organism to be tested was inoculated into 5.0 cc. of Todd-Hewitt broth containing 0.1 cc. of defibrinated rabbit blood. After incubation for 22 hours, 0.05 cc. of the culture was transferred to 5.0 cc. of Todd-Hewitt broth and incubated at 37° C. for 18 to 20 hours. After this period of growth, two drops of 0.01 per cent phenol red solution were added to the culture and the pH was adjusted to pH 7.0-7.5 with 1N NaOH. The culture was then centrifuged and the supernate tested undiluted and in dilutions of 1:5, 1:10, 1:20, and 1:30. The test system consisted of 1.0 cc. of the culture supernate diluted in buffered saline, 1.0 cc. of the 0.6 per cent Fraction I solution, and 0.2 cc. of the thrombin solution. The tubes were incubated in a water bath at 37° C. for 60 minutes, and the end-point was taken as that dilution of culture supernate which produced complete lysis of the fibrin clot. In order to avoid the possible effect of variations in the culture media on streptokinase production, a single lot of broth was used throughout.

Case material: The case material employed in this study was derived from an epidemic of scarlet fever in young adult males at the Great Lakes Naval Training Station, and all patients were admitted between February 26, 1946, and May 2, 1946. With the cooperation of the U. S. Naval Medical Research Unit No. 4, throat cultures and specimens of serum were obtained from these patients on admission to the hospital and at weekly intervals thereafter. The strains of streptococci isolated from the throat cultures were classified serologically in our laboratory by the precipitin technique of Swift, Wilson and Lancefield (20), and preserved for subsequent study by freezing and drying. The sera were stored at 4° C. Patients developing rheumatic fever were transferred to the Naval Hospital at Dublin, Georgia, and late specimens of serum were obtained from these individuals. Summaries of the clinical records of these patients were provided by the medical officers of Naval Research Unit No. 4.⁴

As indicated by serological studies of the streptococcal strains isolated, three types

⁴ The authors are indebted to Lt. Comdr. John R. Seal, MC, USN, and the personnel of the U. S. Naval Medical Research Unit No. 4 for collection of sera and cultures and for supplying the clinical data on the patients. Dr. Robert F. Watson and Dr. Rebecca C. Lancefield represented the Hospital of The Rockefeller Institute for Medical Research in setting up this cooperative project.

of group A hemolytic streptococci (Types 17, 19, and 30) accounted for the great majority of the infections, but at least three other types (Types 1, 3, and one or more non-typeable strains) were involved in the epidemic. Individual isolation of the patients was not feasible, and the presence in the same ward of individuals infected with different types of streptococci resulted in a high incidence of cross infection. This occurrence of cross infection, together with other variables such as treatment and response to treatment, have been taken into consideration in the grouping of the case material chosen for serological studies. Cases were excluded from the study if clinical and bacteriological data were incomplete. Out of a total of 380 cases studied in the epidemic, detailed antibody studies were carried out on 90, grouped according to the following scheme:

Untreated, uncomplicated scarlet fever:

Group I: 17 patients from whom only a single type of group A hemolytic streptococcus was isolated. Although the failure to isolate more than one type of streptococcus from the serial throat cultures does not entirely eliminate the possibility of cross infections in these patients, it reduces the likelihood that clinically significant cross infections occurred.

Group II: 12 patients from whom more than one type of group A hemolytic streptococcus was isolated during the first two weeks of illness.

Penicillin-treated scarlet fever:

Group III: 17 patients from whom group A hemolytic streptococci could not be isolated after the initiation of treatment. Thus, from the bacteriological point of view, this group comprises those patients successfully treated with penicillin.

Group IV: 21 patients who showed persistence of, or recurrence of, a positive culture for group A hemolytic streptococcus of the same or different type.

Scarlet fever followed by rheumatic fever:

Group V: 23 cases. This group is not large enough to justify subdivision for purposes of analysis into sub-groups according to occurrence of cross infection and presence or absence of penicillin therapy. A majority of the patients were treated with penicillin, but none fell into the group of "successfully" treated cases comparable to Group III in which organisms disappeared permanently from the throat after the initiation of therapy. The selection of these rheumatic fever patients was of necessity based on rather rigid criteria, similar to those set down by other investigators. Unequivocal evidence of at least three of the following clinical or laboratory manifestations of rheumatic fever was required: arthritis or arthralgia; fever (subsequent to the initial streptococcal infection); elevated erythrocyte sedimentation rate; prolongation of the P-R interval (over 0.20 seconds); other electrocardiographic abnormalities; pericarditis; occurrence of valvular lesions as evidenced by characteristic murmurs. As a result of the use of these criteria, borderline and doubtful cases are not included. The objection that only the more severe cases of rheumatic fever have been accepted is perhaps valid, although in general these patients had relatively mild,

TABLE I

Detailed Bacteriological and Antibody Data on 90 Cases of Group A Hemolytic Streptococcal Infection

Group	Case	Streptococcus type*					Antistreptokinase† titer					Antistreptolysin O† titer					Gamma globulin grams per cent				
		0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.
I	49	30	30	30	30		40	40	40	80		250	300	300	300		0.72	0.86	0.86	0.89	
	123	30	30	30	30	30	40		80	160	160	150		200	200	150	0.83		1.05	1.05	0.98
	192	30	30	30	30		40	80	320	320		75	100	150	150		0.77	0.72	0.81	0.92	
	198	30	30	30	30		10	10	320	80		300	250	200	600		1.17	1.22	1.14	1.50	
	279	30	30	30	30	NC	80	80	160	320	320	300	250	250	250	250	1.14	0.98	1.08	1.22	1.14
	290	30	30	30	—		40	80	160	160		150	400	300	250		0.81	1.17	1.17	1.14	
	304	30	30	30	—	—	40	80	80	80	80	100	150	100	100	100	0.72	0.83	0.89	0.81	0.81
	11	30	NC	—	—		40	80	80	80		50	100	150	150		0.72	0.75	0.81	0.81	
	324	30	30	30	NC		160	160	640	640		150	200	250	250		0.77	0.77	0.81	0.89	
	63	19	19	19	19	19	10	20	40	40	40	150	150	200	250	200	0.83	0.92	0.92	0.98	0.89
	108	19	19	NC	19	19	0	40		160	160	0	100		200		0.89	0.89		0.92	0.98
	377	19	19	19	19		40	40	160	320		75	100	150	200		0.70	0.72	0.83	0.89	
	295	19	19	19	NC		20	40		40		50	100		100		0.64	1.17		0.92	
	316	19	19	19	19		160	320	640	640		75	100	150	100		0.77	0.95	0.75	0.86	
	131	17	17	17	17		20	320	320	320		25	250	200	250		0.62	0.72	0.77	0.98	
	288	3	3	3	—		40	40	40	40	80	150	250	400	500	100	0.83	0.98	1.00	1.14	0.89
	310	1	1	1	1		160	160	160	160		150	150	200	250		0.81	0.95	1.00	0.95	
	Average						55	99	216	214	140	129	184	213	241	150	0.81	0.91	0.93	0.99	0.95
II	55	17	30	30	19		40	160	640	640		25	25	50	50		0.72	0.83	0.89	1.05	
	97	30	30	17	—	NC	160	160	640	1280	640	100	100	150	250	200	0.95	1.08	1.38	1.55	1.38
	99	30	30	1	1		0	40	80	80		0	0	25	25		0.72	0.81	0.95	0.95	
	58	30	30	17	17		160	320	320	320		150	200	150	150		0.92	1.00	1.08	1.14	
	125	17	17	3	NC	30	20	80	320		320	75	250	500		800	0.77	0.89	1.00		1.50
	158	30	30	—	1	1	40	40	80	80	80	200	250	300	250	250	0.70	0.81	0.77	0.77	0.81
	222	30	19	1	1		80	80	320	320		150	200	400	250		0.83	0.83	1.08	1.22	
	235	3	3	30	30	30	40	80	80	80	160	100	150	200	250	300	0.81	0.89	1.05	1.05	1.14
	265	19	17	17	17		160	160	320	320		300	500	500	500		0.77	0.92	1.08	1.08	
	266	17	19	—	—	—	40	160	160	160	160	300	300	300	300	300	1.00	1.14	1.38	1.22	1.22
	208	17	17	1	1		80	320	640	320		250	250	300	250		1.00	1.22	1.44	1.41	
	14	17	NC	17	19	1	0	20	160	80	80	50	75	500	600	600	0.72	0.92	1.31	1.28	1.08
	Average						68	135	313	334	240	141	191	281	261	408	0.83	0.95	1.12	1.17	1.20
III	41	30	—	—	—		80	80	80	80		100	200	200	250		1.05	1.38	1.17	1.33	
	280	30	—	—	—		80	80	80	80		400	400	400	400		1.08	1.28	1.17	1.22	
	281	30	—	—	—	—	20	20	20	20	20	100	200	150	150	150	0.72	0.89	1.08	1.00	0.89
	94	19	—	—	—	—	20	20	20	20	20	25	25	25	25	25	0.72	0.77	0.72	0.86	0.77
	191	19	—	—	—		40	80	80	80		200	300	300	300		0.89	0.98	1.14	1.00	
	299	19	—	—	—		80	160	160	160		100	150	150	150		0.77	1.17	1.14	1.28	
	323	19	—	—	—		40	40	40	40		250	300	250	200		0.83	1.05	1.00	1.05	
	151	19	—	—	—		20	20	40	20		50	75	75	150		0.77	0.89	0.92	1.17	
	171	19	—	—	—		20	20	20	10		100	100	150	200		0.89	0.89	1.00	1.17	
	201	17	—	—	—		80	80	160	160		50	50	50	50		0.77	1.00	0.95	1.00	
	202	17	—	—	—		40	40	40	40		150	150	150	150		0.83	1.05	1.11	1.11	
	286	17	—	—	—	—	40	40	40	40	40	150	150	200	200	200	0.72	0.77	0.77	0.86	0.89
	261	17	—	—	—	—	20	20	40	20	20	75	200	150	150	150	0.77	1.22	1.08	1.05	1.05
	275	17	—	—	—		80	160	160	160		100	250	150	150		0.70	0.89	0.81	0.81	
	292	3	—	—	—	—	80	80	80	80	80	100	100	150	150	150	0.89	1.00	1.11	1.05	1.17
	258	3	—	—	—	—	40	20	20	20	40	50	100	100	100	100	0.67	0.67	0.77	0.77	0.83
	184	1	—	—	—	—	40	40	40	40	80	150	100	100	100	100	0.67	0.77	0.83	0.77	0.77
	Average						48	59	66	63	43	126	168	162	169	125	0.81	0.99	0.99	1.02	0.92

TABLE I—*Concluded*

Group	Case	Streptococcus type*					Antistreptokinase† titer					Antistreptolysin O† titer					Gamma globulin grams per cent				
		0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.	0 wk.	1 wk.	2 wks.	3 wks.	4-6 wks.
IV	283	19	—	30	30	—	160	320	320	640	640	50	25	50	50	50	0.67	0.67	0.75	0.75	0.77
	289	17	17	—	17	17	20	80	640	640	640	50	200	1200	1200	1000	0.89	0.89	1.38	1.28	1.17
	300	17	—	17	—	—	20	40	40	80	—	100	100	150	150	—	0.67	0.67	0.72	0.67	—
	180	30	—	19	19	19	80	80	80	80	640	25	25	50	100	100	0.67	0.67	0.77	0.62	0.98
	309	30	—	—	19	19	160	320	320	320	320	100	100	150	150	200	0.77	0.83	0.77	0.75	0.75
	196	30	—	30	19	19	40	80	80	1280	2560	50	100	100	150	200	0.67	0.72	0.67	0.98	1.05
	334	19	—	30	—	—	80	80	160	160	640	150	150	250	250	250	0.89	1.17	1.11	1.17	1.17
	206	30	—	—	17	—	20	40	40	40	80	200	150	300	200	300	0.95	0.98	1.11	1.22	1.17
	267	30	—	30	—	—	80	80	80	80	—	250	250	250	250	—	0.75	0.89	0.95	0.98	—
	269	17	—	—	17	17	160	320	160	160	160	100	200	100	100	150	0.72	0.81	0.89	0.81	0.83
	249	30	—	3	—	—	320	640	640	640	640	250	250	300	300	400	0.62	0.67	0.67	0.72	0.77
	379	17	17	17	17	—	20	40	80	80	—	100	200	400	400	—	1.25	0.95	1.00	1.00	—
	54	17	17	17	17	—	40	80	160	160	—	100	150	150	200	—	0.62	0.59	0.72	0.75	—
	56	17	17	17	17	17	40	40	40	80	160	100	100	100	150	250	0.62	0.75	0.75	0.83	0.81
	68	30	30	17	NC	17	160	40	160	640	640	50	100	100	100	150	0.77	0.77	1.00	1.22	1.28
	71	19	19	19	1	—	0	0	320	320	—	25	25	150	200	—	0.81	0.95	1.11	1.11	—
	73	30	17	1	1	—	40	40	80	160	160	100	100	150	250	300	0.95	1.05	1.22	1.25	1.11
	126	19	17	17	—	—	320	320	320	—	—	200	250	300	—	—	0.75	0.75	0.67	—	0.95
	133	30	17	17	17	17	10	10	40	40	40	25	50	100	150	150	0.81	0.81	0.89	1.17	1.05
	305	30	—	19	—	—	160	320	160	160	160	200	300	300	300	400	0.75	0.81	0.81	0.83	0.83
	209	19	—	17	17	—	80	80	160	2560	—	100	100	100	500	—	0.77	0.81	0.83	1.44	—
Average							96	145	194	416	541	111	139	226	257	286	0.77	0.82	0.89	0.98	0.98
V	13	30	—	—	—	1	80	80	320	1280	2560	150	250	300	300	300	1.02	1.28	1.44	2.48	2.64
	16	19	19	19	1	3	20	20	160	320	320	100	100	100	200	200	0.89	1.05	1.22	1.28	1.33
	23	17	—	17	17	17	40	160	640	640	640	150	250	300	700	300	0.95	1.02	1.38	1.33	1.28
	30	3	—	—	19	19	40	320	80	80	80	150	400	2000	3000	2000	0.95	1.11	1.67	1.38	1.55
	51	19	19	17	17	17	40	40	80	320	640	300	300	500	600	600	1.05	0.95	1.38	1.72	1.55
	52	17	17	17	3	3	160	160	320	640	640	100	200	250	400	1600	0.81	0.89	1.05	1.38	1.72
	65	19	19	19	—	—	20	20	160	640	640	250	250	300	700	500	0.89	1.05	1.38	1.83	1.67
	88	17	17	—	17	17	20	10	80	80	80	25	25	300	400	400	0.92	0.98	1.67	1.96	1.55
	92	1	1	30	17	—	40	80	160	160	640	100	150	300	500	1000	0.72	0.95	1.17	1.44	1.97
	120	30	30	30	—	—	80	80	320	640	1280	150	200	200	200	250	0.98	1.00	1.17	1.20	1.88
	234	19	19	—	—	—	80	160	320	640	640	1200	1600	1400	1200	1400	1.17	1.28	1.22	1.55	1.44
	247	—	—	17	17	17	640	640	640	1280	1280	150	200	200	200	200	0.92	1.08	0.95	1.08	1.08
	251	—	30	30	—	—	0	0	80	640	1280	200	200	200	500	700	0.81	0.81	0.95	1.83	1.88
	61	30	30	30	3	3	320	320	2560	2560	2560	100	150	250	300	400	0.67	0.75	1.00	1.00	1.05
	42	NT	NT	NT	NT	NT	40	80	80	80	80	100	150	200	150	150	0.77	0.77	0.95	1.00	1.17
	45	17	17	—	—	30	40	80	160	20	160	100	100	150	100	250	0.77	0.83	1.11	0.98	1.00
	181	30	—	—	17	17	40	160	320	320	320	200	600	1400	2000	2000	0.70	0.75	1.00	1.17	1.14
	175	19	—	17	17	—	0	0	10	40	80	250	250	250	250	250	1.05	1.22	1.14	1.14	1.22
	164	17	17	17	—	—	40	320	320	320	320	300	700	800	600	700	1.33	1.99	2.21	2.21	2.54
	173	30	3	30	17	—	160	160	320	320	640	100	150	150	250	250	1.17	1.33	1.44	1.28	1.17
	217	17	—	17	—	17	40	160	320	640	1280	250	300	300	500	800	0.89	1.05	1.00	1.17	1.55
	337	—	—	—	19	19	20	20	40	80	160	200	200	200	200	250	0.81	0.95	0.95	1.11	1.11
	44	30	17	17	—	30	160	160	160	320	640	250	200	500	600	700	1.05	1.44	1.38	1.38	1.50
Average							92	140	332	524	737	212	301	459	602	661	0.92	1.06	1.25	1.43	1.52

* NCO No culture obtained.

— No hemolytic streptococci.

NT Group A, no type.

† Antistreptokinase titers of less than 10, and antistreptolysin O titers of less than 25 are listed as 0.

monocyclic attacks, and few of them would be considered severe according to the usual clinical standards.

Experimental Results

The results of antistreptokinase, antistreptolysin O, and gamma globulin determinations on the sera of the 90 patients are recorded in detail in Table I. Although certain facts are apparent from inspection of the table, the significant

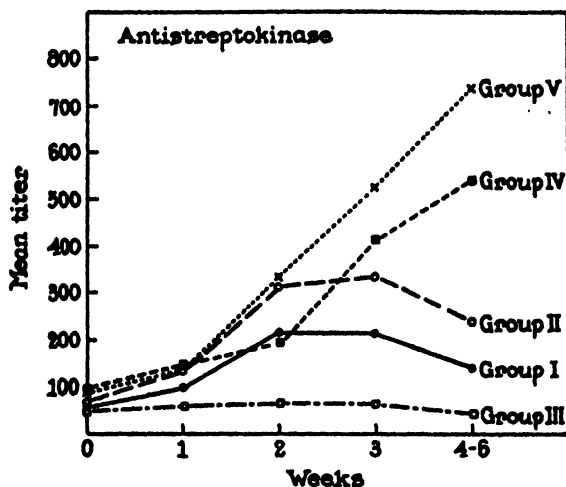


FIG. 1. Mean antistreptokinase titers at weekly intervals from time of admission to hospital.

Group I: Uncomplicated scarlet fever; single type of streptococcus.

Group II: Uncomplicated scarlet fever; multiple types of streptococcus.

Group III: Penicillin-treated scarlet fever with disappearance of organism from throat.

Group IV: Penicillin-treated scarlet fever with persistence or reappearance of streptococci in throat culture.

Group V: Scarlet fever followed by rheumatic fever.

conclusions to be drawn from the large mass of data are better illustrated by graphic treatment. The complete data are given for reference in conjunction with the figures which summarize the various aspects to be discussed.

In Figures 1-3, the arithmetic means of the three determinations are plotted against the week of disease. It will be seen at once from an inspection of these figures that, in general, the pattern of response within each group is similar in all three determinations. For example, the group of rheumatic fever patients (Group V) has consistently higher antibody titers and gamma globulin levels throughout the period studied than any of the other groups. The possible

significance of this finding will be considered in detail below. In addition, there is a consistent difference between the degree of response of those untreated

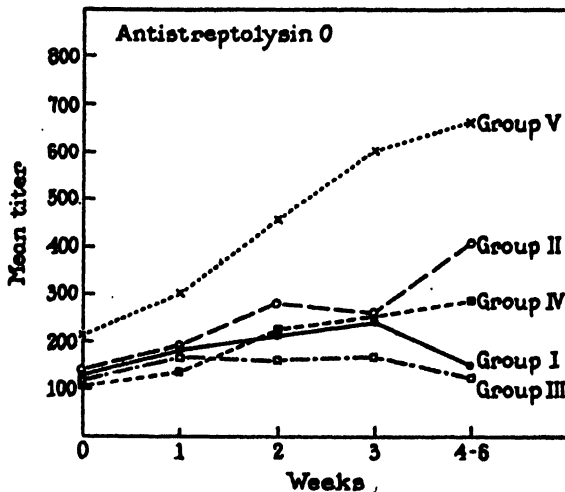


FIG. 2. Mean antistreptolysin titers at weekly intervals from time of admission to hospital. Groups as in Figure 1.

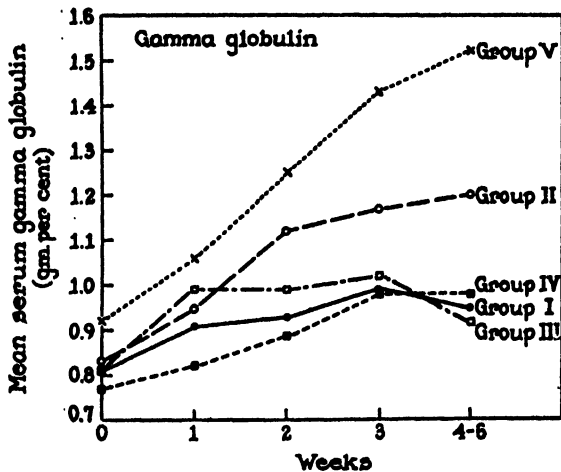


FIG. 3. Mean serum gamma globulin levels at weekly intervals from time of admission to hospital. Groups as in Figure 1.

scarlet fever patients with single type infections (Group I) and those with multiple type or cross infections (Group II). The higher average response of the latter group suggests that the occurrence during infection of more than one type of streptococcus may augment the antibody response.

The failure of production of antibodies to streptokinase and streptolysin O in those patients "successfully" treated with penicillin (Group III) is apparent from inspection of Tables I and II, and of Figures 1 and 2. In no case was there a significant rise in antistreptokinase, while only eight of the 17 patients showed a significant, though minimal, response of antistreptolysin O. It should be remembered, however, that because of the differences in dilution increments used in the measurement of the two antibodies, a significant (or two-tube) rise in antistreptolysin O implies a smaller actual increase in antibody than in the case of antistreptokinase. Similar observations on the decreased antistreptolysin O response in patients with hemolytic streptococcal infections treated with large doses of penicillin have been reported by Rantz, Boisvert, and Spink (21), and by Weinstein and Tsao (22). In contrast to the flattened curves seen in Figures 1 and 2 for this group (Group III), the average gamma globulin levels (Figure 3) show a rise comparable to those of Groups I and IV.

TABLE II

Percentage Incidence of Significant Rises in Antistreptokinase and Antistreptolysin O Titers

Group	No. of cases	Per cent showing significant rises in—			
		ASK	ASO	ASK and ASO	Either ASK or ASO
I	17	65	76	53	88
II	12	75	58	42	92
III	17	0	47	0	47
IV	21	71	76	62	90
V	23	87	83	74	96

This suggests that the antibody response as a whole may not be greatly suppressed by the administration of penicillin. The two specific antibodies measured are directed against extracellular products elaborated by growing streptococcal cells, and may represent special cases with respect to the effect of penicillin on antibody production.

The proportion of patients in each group showing significant rises in antistreptokinase and antistreptolysin O titers may be seen in Table II. With the exception of Group III, the incidence of significant responses of the two antibodies is similar within each group. The percentage difference in Group III is probably more apparent than real for the reason mentioned before. As demonstrated by the data given in the last two columns of Table II, patients with a significant antibody response to one antigen did not necessarily have a corresponding response to the other. The percentage of patients showing significant simultaneous rises in both antibodies is much smaller than the percentage showing a rise in either antistreptokinase or antistreptolysin O. No criterion for significance of an increase in gamma globulin has yet been rigidly defined,

although with this method the normal range has been found to be about 0.67–1.0 gm. per cent (18).

The occurrence of consistently higher mean values for the two antibodies as well as for the gamma globulin levels in that group of patients with rheumatic fever (Group V) as compared with the other groups is worthy of special reference. Although the number of uncontrollable variables inherent in a study of this kind may cast some doubt on the validity of applying statistical methods, the results of statistical analysis of these data suggest that the observed differences are significant. For example, in the case of the mean value for the serum gamma globulin at three weeks after the onset of scarlet fever, the observed difference between the rheumatic fever patients and the entire non-rheumatic

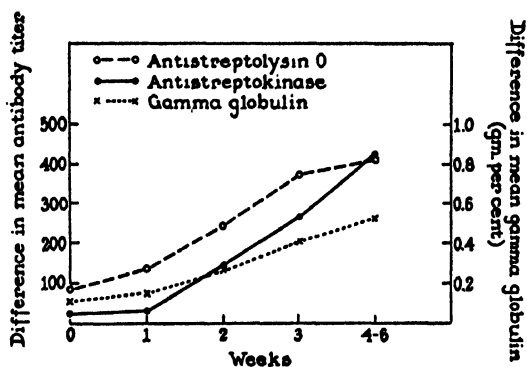


FIG. 4. Difference in antistreptokinase, antistreptolysin and gamma globulin levels between the rheumatic fever Group (Group V) and the non-rheumatic Groups (Groups I-IV).

group is four times its standard error. According to statistical theory, the probability of this difference occurring by chance is 1 in 15,000.

There are additional considerations which appear to support the interpretation that the observed difference is significant. To provide graphic representation of the observations, the mean weekly value of each of the three determinations for the 67 scarlet fever patients in Groups I-IV have been subtracted from the corresponding mean values for the rheumatic fever patients (Group V) and the differences plotted in Figure 4. It will be seen that in the case of antistreptokinase and antistreptolysin O the absolute differences, as well as the rate of increase of the differences, are comparable. Although not directly comparable because they are expressed in different units, the difference in the gamma globulin levels shows a rise similar to that of the antibodies. The mechanism and technical procedures involved in the three determinations vary widely. It is all the more striking, therefore, that the differences observed are of the same order in the case of each test.

The possibility must be considered that the arithmetic means for the various determinations may be unduly influenced by the occurrence of both very high and very low values with resultant exaggeration of minor differences. Consequently, frequency distributions were analysed for the three determinations, and are illustrated by the chart for antistreptokinase reproduced in Figure 5. At the time of the initial bleedings, the frequency distribution of the titers is remarkably similar for the two groups. By the end of 21 days, however, the two distributions have lost their similarity, and the one for the rheumatic fever group has shifted more to the right than has the one for the non-rheumatic fever group. Similar analyses of the antistreptolysin O and gamma globulin data yield comparable results. Thus this treatment of the data appears to support the significance of the differences between the rheumatic and non-rheumatic groups that was first suggested by the mean values of the various determinations.

In vitro production of streptokinase: Because previously published work (19) suggested that the antistreptokinase response of the patient was somewhat proportional to the quantity of streptokinase produced *in vitro* by the infecting organism, a number of group A hemolytic streptococci isolated from patients in this investigation were studied for their ability to produce streptokinase. Ninety-four assays were performed, using 62 different strains. Supernates of ten Type 1 and ten Type 3 streptococcal cultures were tested, and, without exception, all failed to lyse a standard clot even when tested undiluted. These two types were far less common during the epidemic than were the other types, so that untreated patients from whom only a Type 1 or a Type 3 streptococcus was isolated were few in number, and only two are represented in Table I. Neither has a significant antistreptokinase response. Forty-two group A hemolytic streptococci of Types 17, 19, and 30 were tested, and all produced measurable amounts of streptokinase. The supernates of two strains could be titrated only to a dilution of 1:5; all the rest titrated to dilutions of 1:10 or 1:20. There was no difference in ability to produce streptokinase between those organisms isolated from patients with rheumatic fever, and those from patients who did not develop rheumatic fever.

DISCUSSION

The results of the present studies provide no evidence that those patients who develop rheumatic fever following group A hemolytic streptococcal infections have a pattern of antibody response to streptokinase that differs from the pattern of their response to other antigenic stimuli. On the contrary, the quantitative differences in antistreptokinase production which are observed in a comparison of the rheumatic and non-rheumatic patients are paralleled by comparable differences in the production of a second antibody, antistreptolysin O, and in the increase in serum gamma globulin. The data on gamma globulin

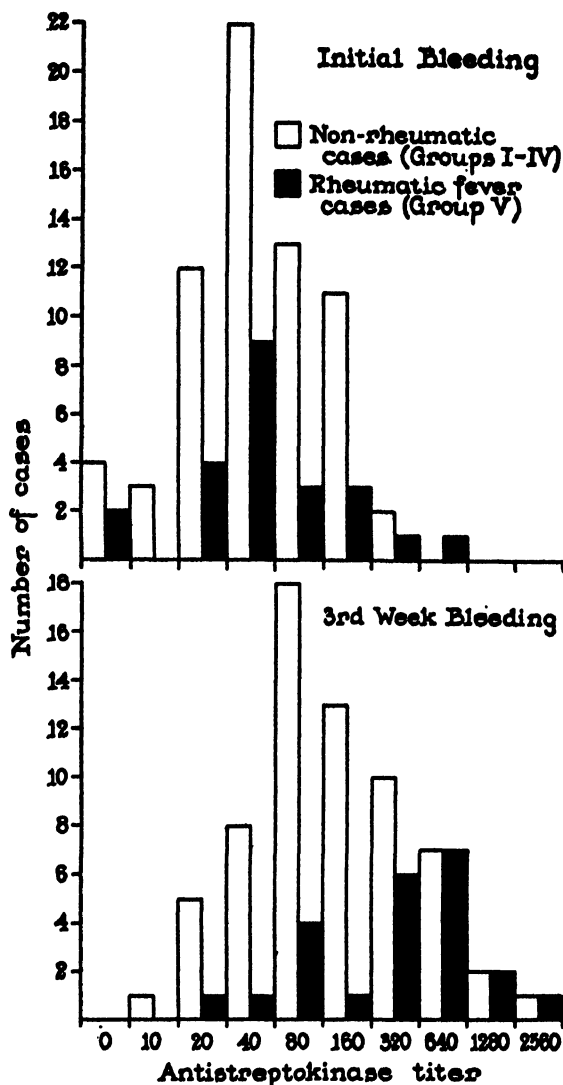


FIG. 5. Comparison of distribution of antistreptokinase titers of rheumatic (group V) and non-rheumatic (Groups I-IV) Groups at time of admission with scarlet fever and after three weeks.

suggest that the greater production of the two antibodies in those patients who developed rheumatic fever is a reflection of a general augmentation of antibody formation.

It is important to point out that in the present study the increased antibody

production is made apparent only by the collective analysis of the two groups of cases, and no basis is provided for differentiating between individual patients. Thus, some of the patients with uncomplicated scarlet fever showed a greater rise in antibody titer than certain of the rheumatic fever patients, and a few of the latter have relatively insignificant increases.

Quantitative differences in production of antibody have not been emphasized in previous investigations of group A hemolytic streptococcal infections in which rheumatic and non-rheumatic patients have been compared (23, 24, 25, 26, 27). In a recent study, Rothbard, Watson, Swift, and Wilson (28) found that the average antistreptolysin O response, as indicated by the ratio between the maximal titer and initial titer, was greater in patients who developed rheumatic fever following a streptococcal infection than in those who had uncomplicated infections. However, they found in addition that a group of patients with purulent complications showed a much greater average response than either of the other two groups. The fact that the differences described in the present study are so readily apparent may be in some degree due to the homogeneity of the case material. These patients were selected from a single epidemic of scarlet fever occurring within a period of two months, and a relatively small number of streptococcal strains was involved. Furthermore, the patients represented an unusually uniform sample of the population, since all were males between the ages of 17 and 27; 82 (91 per cent) of the men were included in the age group of 17 to 20 years. Accordingly, some of the uncontrollable variables commonly present in this type of study were eliminated, and it is reasonable to suppose that small differences of the sort described might become apparent under these conditions.

The possible significance of the apparent enhancement of antibody formation in the rheumatic subjects is difficult to assess. Conceivably it might mean that on the average these patients received a greater antigenic stimulus in the form of a more serious or extensive streptococcal infection, although there is nothing in the clinical histories to support this point of view. A second possible interpretation is that persons susceptible to rheumatic fever may in general respond to a given stimulus with greater production of antibody. It is well known, for example, that individual differences in degree of antibody formation occur among experimental animals injected with identical amounts of antigen. It is also worthy of note that even at the onset of the streptococcal infection, those patients who later developed rheumatic fever had, on the average, higher antibody titers and higher gamma globulin levels than did those who did not develop rheumatic fever. Regardless of the interpretation one wishes to put on the results, it seems premature and fruitless to attempt to reconcile them with any of the current theories concerning the pathogenesis of rheumatic fever.

SUMMARY AND CONCLUSIONS

1. A procedure for the quantitative determination of antistreptokinase has been employed to follow the antibody response of patients with scarlet fever, including those who developed rheumatic fever. Antistreptolysin O titers and gamma globulin levels on the same sera are presented for comparison.

2. There is suggestive evidence that the presence of two or more types of streptococci during an infection calls forth a greater antibody response than does the presence of only a single type.

3. Early and effective penicillin therapy which removed the infecting organism promptly from the nasopharynx either prevented entirely or greatly decreased the expected antibody response to streptokinase and streptolysin O. No effect was apparent on the total antibody response as measured by the serum gamma globulin.

4. Development of the rheumatic state is not accompanied by a pattern of antistreptokinase response that differs significantly from the pattern of the general immune response in the same state.

5. Of the patients included in this study, it would seem that on the average those who developed rheumatic fever as a sequela to a streptococcal infection exhibited a greater antibody response than those who did not develop rheumatic fever.

Acknowledgment

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THE OCCURRENCE OF NUCLEASES IN CULTURE FILTRATES OF GROUP A HEMOLYTIC STREPTOCOCCI

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The enzymes which are released into the environment by pathogenic microorganisms are of importance in a consideration of the mechanism of disease processes, since they may play a rôle in the virulence and invasiveness of the bacteria as well as in specific tissue injuries. In this connection, it is of interest that certain of the bacterial exotoxins have recently been demonstrated to be enzymatic in character. In the case of group A hemolytic streptococci, a number of extracellular products possessing a wide variety of biological activities have been recognized. Some of these substances, for example, streptolysin O, streptokinase, and streptococcal proteinase, have been subjected to intensive study, and a considerable body of information is available concerning their chemical and biological properties and their possible relationship to the pathogenesis of streptococcal disease. Because of the value of this type of study and the probability that other hitherto unrecognized substances accumulate in the environment of growing streptococci, the enzymatic activities of culture supernates of these organisms were investigated further. In the course of these studies, it was found that both ribonuclease and desoxyribonuclease occur with great regularity in culture media in which group A hemolytic streptococci are grown. The present paper deals with the extracellular occurrence and enzymatic activity of the two nucleases released from streptococcal cells during growth in fluid media.

Materials and Methods

Strains of Group A Hemolytic Streptococci.—Thirty-six different strains of group A hemolytic streptococci were studied for their ability to form nucleases. Sixteen serological types were represented and strains were included with special biological characteristics such as formation of large amounts of streptokinase or streptococcal proteinase, formation of hyaluronidase, failure to form erythrogenic toxin or streptolysin S, and requirement of the purine growth factor described by Wilson (1). A summary of the strains employed is given in Table I. The strains are designated by the numbers used in this laboratory, and the serological type is indicated in parentheses.

Bacteriological Media.—Three different media were employed: (a) Todd-Hewitt buffered broth (2), (b) neopeptone dialysate broth prepared according to the method of Dole (3), and (c) the partially defined medium developed by Adams and Roe for growth of pneumococci (4).

Preparation of Cultures.—16 to 18 hour cultures were used for the most part. The bacterial cells were removed by centrifugation, and in many experiments the supernates were tested without sterilization. While this procedure required that certain precautions be observed in

the handling and disposal of the material, it proved simpler than attempting to sterilize by filtration as a routine. However, it was found that the culture supernates could be passed through Coors P3 filters without loss of enzymatic activity, and this method was used to obtain bacteria-free filtrates.

Measurement of Ribonuclease.—A turbidimetric method was employed as the routine procedure for measuring ribonuclease activity, since the other methods available are not readily adaptable to use with nutrient broth. The method is based on the fact that under the

TABLE I

Strains of Group A Streptococci Tested for Production of Ribonuclease and Deoxyribonuclease

Special biological characteristics	Strain designation and serological type
Produces high yield of streptokinase	H105 (Tillett's "Co"; undesignated new type)
Produces high yield of streptococcal proteinase	B220 (Elliott's strain 5797; type 8 T antigen, no M antigen identified)
Produce extracellular hyaluronidase	C748 (type 4); B247 (type 22)
Stock strain for production of streptolysin O	D58 (Colebrook's strain "Richards," type 3)
Produce no streptolysin S	C439, C440, C441 (Colebrook's strains, all type 12)
Widely used for production of erythrogenic toxin	NY5 (type 12*)
Produces no erythrogenic toxin	C998 (Todd's "Cooper 3122," type 3)
Require Wilson's purine growth factor	C811, C812, C813, C272 (all type 19); C660 (type 19, no T antigen)
Do not require Wilson's purine growth factor	C817, C820, S24 (all type 19); C655 (type 19, no T antigen)
Recently isolated from scarlet fever patients	1GL19 (type 3); 1GL21 (type 17); 1GL4 (type 19); 1GL49 (type 30); 1GL22 (type 30)
Stock strains of various serological types	T1 (type 1); C203 (type 3, types 1 and 3 T antigens); S43 (type 6); S23 (type 14); J17E (type 17); J17F (type 26); T28 (type 28); D23 (type 29); D24 (type 30); London (undesignated new type)

* Originally classified as type 10 on the basis of typing by agglutination.

conditions employed the end-products of the reaction are soluble in acid, in contrast to the insolubility of the undigested nucleic acid. Ribonucleic acid from yeast was purified by the method of Kunitz (5). A stock solution containing 5 mg. per cc. was prepared by adding to an aqueous suspension of the nucleic acid just enough 1 N NaOH to cause complete solution. A solution so prepared has a pH of approximately 5.0 and is quite stable at refrigerator temperatures. A fivefold dilution of the stock solution in 0.025 M veronal buffer pH 7.5 is used as substrate for the test. The substrate solution is mixed with an equal volume of culture supernate and incubated in a water bath at 37°C. At intervals, 1.0 cc. of the reaction mixture is removed, mixed with 1.0 cc. of 1 N HCl, and the optical density of the resulting precipitate determined in a Coleman junior spectrophotometer at wave length 425 mμ. Turbidity controls for each of the reagents are employed. At appropriate enzyme concentrations the decrease in turbidity is linear with time over a period of 20 minutes. Contrary to expectation

on the basis of published data concerning the instability of yeast nucleic acid at alkaline pH, the substrate shows no spontaneous loss of acid precipitability in the absence of enzyme. Even after several hours at 37°C. and pH 7.5, the optical density after addition of HCl is identical with that of freshly prepared solutions.

Measurement of Desoxyribonuclease.—For routine testing of desoxyribonuclease a turbidimetric method identical with the ribonuclease method was used, except that the substrate solution was a 0.1 per cent solution of sodium desoxyribonucleate from calf thymus in 0.025 M veronal buffer pH 7.5 containing 0.01 M $MgSO_4$. In general, because of the higher activity of this enzyme, it was necessary to use dilutions of the culture filtrate to obtain linear rates of decrease in turbidity. Control tests of undigested substrate frequently give fibrous precipitates on the addition of acid which interfere with turbidimetric readings, but the results of enzymatic tests are unequivocal.

The turbidimetric method was supplemented by the more quantitative viscosimetric method previously described for estimating desoxyribonuclease activity (6). By this means, it was possible to make quite accurate comparisons of the potency of streptococcal nuclease with nuclease from other sources, e.g., beef pancreas.

EXPERIMENTAL

Nuclease Activity of Culture Supernates.—All thirty-six strains studied produced both ribonuclease and desoxyribonuclease, and no relation was detected to the other biological characteristics of the strains. Although there were quantitative differences in the amount of the two enzymes elaborated by different strains, the variations were not great and were not studied in detail. Supernates of cultures in neopeptone dialysate broth uniformly showed higher activity than those in Todd-Hewitt broth. This difference is not referable to greater growth of the organisms in dialysate broth, but is due, in part at least, to the presence of inhibitory substances in the Todd-Hewitt broth.

Ribonuclease activity was relatively low and dilution of the culture supernates was not necessary. The results of a test of a typical culture supernate are presented graphically in Fig. 1. The effect is comparable to that obtained with 0.01 $\mu g.$ of crystalline ribonuclease from beef pancreas as tested by the same method. In comparison with ribonuclease, desoxyribonuclease activity was surprisingly high. The data plotted in Fig. 1 show that a fiftyfold dilution of the culture supernate was required to give a decrease in acid precipitability comparable to that obtained with ribonucleic acid and undiluted culture supernate. The discrepancy in the relative activity of the two enzymes is probably even greater than that indicated, since the desoxyribonucleic acid has a much higher molecular size than the ribonucleic acid and presumably more enzymatic action is required to reduce the former to acid-soluble products. Evidence for this view was obtained in an experiment in which partial breakdown products of desoxyribonucleic acid, more nearly comparable in molecular size to the ribonucleic acid, were used as substrate in the turbidimetric test. At the same enzyme concentration, the rate of decrease in acid precipitability of the degraded substrate was four times as rapid as that of the intact nucleic acid.

The results of determination of desoxyribonuclease activity by the more

sensitive viscosimetric method confirm the findings of the turbidimetric test. With the same culture supernate as that employed in the test recorded in Fig. 1,

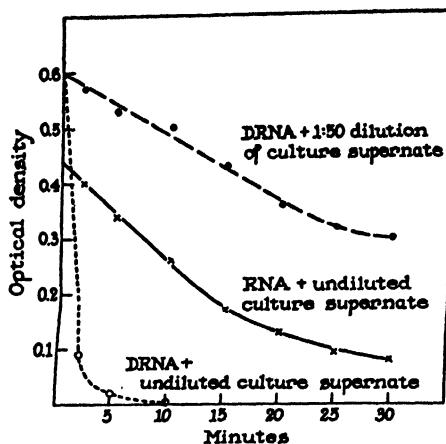


FIG. 1. Ribonuclease and desoxyribonuclease activity of culture supernate of strain C817 grown in neopeptone dialysate broth. DRNA indicates desoxyribonucleic acid, and RNA ribonucleic acid.

TABLE II
Relative Desoxyribonuclease Activity of Various Source Materials

Material	Units per cc.
Beef pancreas—0.25 N H ₂ SO ₄ or aqueous extract*	3300-3500
Swine pancreas—aqueous extract*	1000-2000
Group A hemolytic streptococci—culture supernate	200-400
Type IIR pneumococcus—culture supernate	1-2
Beef spleen—aqueous extract*	<1
Calf thymus—aqueous extract*	<1
<i>Escherichia coli</i> †—culture supernate	0
<i>Bacillus subtilis</i> †—culture supernate	0

* Tissue extracted with 2 to 3 volumes of solvent.

† One strain only tested.

0.0025 cc. (0.5 cc. of a 1:200 dilution) contains one unit of enzyme in the terms defined for use with the pancreatic nuclease (6). Thus, the unconcentrated culture supernate contains 400 units per cc., and a final dilution in the viscosimeter of 1:2000 causes a rapid fall in viscosity. With the exception of pancreatic extracts, streptococcal cultures have proved to be the most potent crude preparations of desoxyribonuclease of the various tissue extracts and bacteriological preparations tested in this laboratory. A comparison of the desoxyribonuclease activity of representative source materials is given in Table II.

It will be seen that the activity of the initial extracts of beef pancreas is about eightfold greater than that of streptococcal cultures. On the other hand, extracts of organs such as thymus and spleen have relatively little activity and the cultures of certain other species of microorganisms appear to be wholly devoid of desoxyribonuclease.

Rate of Production of Desoxyribonuclease.—Desoxyribonuclease is released into the medium by streptococci early in the active growth phase. This is demonstrated in Fig. 2, which records the results of a series of quantitative viscosi-

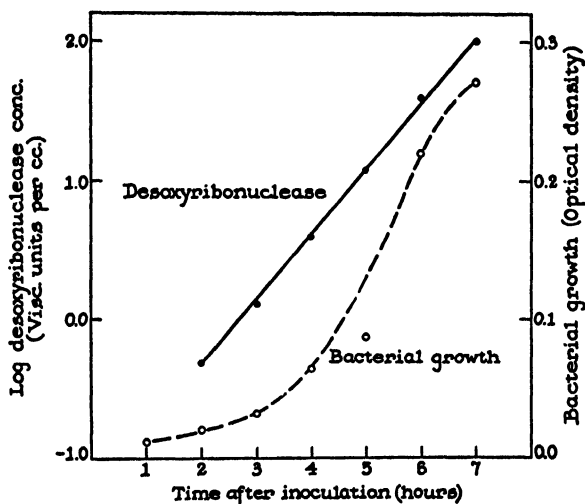


FIG. 2. Rate of increase in concentration of desoxyribonuclease in culture supernate during active growth of group A hemolytic streptococcus (strain H105).

metric determinations of desoxyribonuclease on samples of the supernate of the same culture at intervals after inoculation. A rather large inoculum of washed young cells (2×10^8 organisms per cc.) was used in order to avoid a long lag period. Desoxyribonuclease was detectable in the culture supernate at one hour and was present in accurately measurable amounts at 2 hours after inoculation. Bacterial growth was estimated turbidimetrically. As shown in Fig. 2, the concentration of enzyme increased logarithmically throughout the logarithmic growth phase of the organisms. It appears relatively certain, therefore, that the presence of the enzyme in the culture medium is not merely the result of the degeneration of aging cells.

Effect of Substrate on Nuclease Production.—An attempt was made to determine whether the presence of nucleic acids or enzymatic split products of nucleic acid influences the quantity of nuclease produced during growth. For this purpose it was necessary to use the partially defined medium of Adams and

Roe, since the more complex media contain unknown amounts of nucleotides. Most strains of group A hemolytic streptococci will not grow on repeated transfer in the Adams-Roe medium, but a few strains will give good growth provided moderately large inocula are used. A study of the growth of one strain (D24) by serial transfers in this medium revealed that both ribonuclease and desoxyribonuclease appear in the cultures, although in consistently smaller amounts than in the dialysate broth cultures.

The following substances were incorporated in the Adams-Roe medium at a final concentration of 0.1 to 1.0 mg. per cc.: desoxyribonucleic acid from calf thymus, ribonucleic acid from yeast, enzymatic split products of desoxyribonucleic acid prepared with pancreatic desoxyribonuclease, and enzymatic split products of ribonucleic acid prepared with crystalline ribonuclease. Strain D24 was grown in the presence of these various added substances, and in no case was there any measurable increase in nuclease content of the cultures as compared with control cultures in plain Adams-Roe medium. Furthermore, desoxyribonuclease production was not enhanced by the addition of large amounts of sodium desoxyribonucleate (up to 1 gm. per 100 cc. of culture) to cultures of streptococci in which heavy growth was obtained by continued neutralization of the acid formed in the presence of excess glucose. These experiments suggest that the nucleases, in contrast to streptococcal hyaluronidase (7), are not "adaptive" in character.

Relationship of Nuclease Production to Requirement for a Purine Growth Factor.—Wilson (1) has shown that certain strains of group A streptococci when inoculated into Adams-Roe medium supplemented with horse serum require the addition of a purine factor for growth. Viscous desoxyribonucleic acid did not serve as a source of the growth factor, although it was rendered active by digestion with pancreatic desoxyribonuclease. In a previous paper this finding was interpreted as indicating that these strains of group A streptococci lack the capacity to form an enzyme analogous to pancreatic desoxyribonuclease (6). However, the results of the present studies are not compatible with this interpretation, since several of the strains employed by Wilson have been tested and found not to differ from other strains with respect to desoxyribonuclease formation. Furthermore, a partially purified preparation of the enzyme was prepared from one of Wilson's strains (2884F, designated C811 in Table I) and was shown to be capable of converting calf thymus desoxyribonucleic acid into an active source of the growth factor, just as does pancreatic desoxyribonuclease. It is apparent, therefore, that the failure of the organisms to grow in the horse serum medium with added desoxyribonucleic acid is not referable to an inherent inability to form desoxyribonuclease. However, with the small inocula used (10^{-6} cc.), growth is probably not initiated in the absence of the purine factor and thus no enzyme is formed to convert the desoxyribonucleic acid into an available source of the factor.

DISCUSSION

The data recorded in the present paper indicate that both ribonuclease and desoxyribonuclease are produced by a wide variety of strains of group A streptococci during growth in fluid media. In relative terms, a large amount of desoxyribonuclease is formed; for example, 1 cc. of unconcentrated culture supernate is sufficient to cause depolymerization of several grams of desoxyribonucleic acid. Nothing is known at present concerning the possible rôle of the nucleases in the multiplication of streptococci in host tissues, and it has not been determined whether antibodies which inhibit the enzymes are formed following streptococcal infections. Because of the nature of their action, it seems unlikely that either of the nucleases is capable of damaging living cells, and their action *in vivo* is probably limited to the breakdown of nucleic acids released by tissue cells that have been destroyed by other agents.

The occurrence of the nucleases in culture supernates is not only of theoretical interest but has been shown to be of practical importance in the isolation and purification of other extracellular products, such as streptokinase and proteinase, since the nucleases must be separated from them in the course of purification procedures. Measurement of nuclease activity provides a delicate and simple test for the efficacy of the fractionation procedures employed. Furthermore, since streptococcal desoxyribonuclease is present in relatively large amounts, its preparation in purified form is feasible. In preliminary experiments fractions have been obtained which have a higher specific activity than pancreatic desoxyribonuclease made by the method previously described (6). These streptococcal desoxyribonuclease preparations have the additional advantage of being free of proteolytic activity.

The several enzymes which have been described as being released or "secreted" into the environment by group A hemolytic streptococci have in common the property of attacking substrates of large molecular size. Thus, streptokinase, streptococcal proteinase, hyaluronidase, ribonuclease, and desoxyribonuclease are involved directly or indirectly in the degradation of macromolecules. From one point of view, these enzymes may be interpreted as serving as a "digestive" function; that is, of preparing potential nutrient material so that it can be assimilated by the microorganism. Wilson's studies on the purine growth factor are in accord with this interpretation, since it seems apparent that the action of desoxyribonuclease on desoxyribonucleic acid results in the formation of certain products which the streptococcal cell is able to utilize. In connection with the possible "digestive" nature of these enzymes, it is of interest to compare the known enzymes of streptococcal supernates with those of mammalian pancreatic secretion. A single streptococcal culture supernate can contain a proteinase, a carbohydrase, and the two nucleases, enzymes which are representative of some of the main components of the pancreatic secretion. The analogy can be pursued further, since streptococcal proteinase,

like the pancreatic proteinases, has been shown to be released in the form of an inactive precursor (8).

SUMMARY

1. All of the thirty-six strains of group A hemolytic streptococci tested were found to elaborate ribonuclease and desoxyribonuclease during growth in liquid cultures. Both enzymes are released into the medium.

2. Desoxyribonuclease is consistently produced in greater amount than ribonuclease. The concentration of desoxyribonuclease in the culture increases logarithmically during active growth of the organisms.

3. Under the conditions employed, the presence of specific substrate or enzymatic split products of the substrate did not influence the production of either nuclease.

4. The failure of viscous desoxyribonucleic acid to serve as a source of the purine growth factor required by certain strains of group A streptococci was shown not to be referable to the inability of these strains to form desoxyribonuclease.

5. The determination of nuclease activity provides another criterion for evaluating purification procedures used in the attempted isolation of substances such as streptokinase and proteinase from the supernates of streptococcal cultures.

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PROTECTIVE EFFECT OF HYALURONIDASE AND TYPE-SPECIFIC ANTI-M SERUM ON EXPERIMENTAL GROUP A STREPTOCOCCUS INFECTIONS IN MICE

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PLATES 13 TO 15

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The pathogenicity and invasiveness of group A streptococci for man, as well as for laboratory animals, have been shown to be closely correlated with the presence of the M protein; conflicting evidence, however, has been obtained by several groups of investigators with regard to the rôle of the hyaluronic acid capsule in the manifestation of virulence of these microorganisms.

A number of studies have been carried out in an effort to ascertain the effect of these substances on the pathogenesis of streptococcal infections. Several workers have studied the protective action in mice, infected with group A streptococci, of immune serum containing antibodies directed against the M substance. Others have studied the protective effect of hydrolyzing the capsular material with an enzyme, hyaluronidase. As a result of the antibody studies the M substance has been shown to be essential for the exhibition of virulence, since anti-M antibodies provide active and passive protection of mice against infection with virulent streptococci of homologous type (1-3). On the other hand, the relation of the hyaluronic acid capsule to mouse virulence is not so well defined. While Hirst (4) was able to protect mice infected with encapsulated group C streptococci by treatment with leech extract containing hyaluronidase, he was not able to protect mice infected with group A streptococci; although the capsular materials of both these groups of streptococci have been shown by Seastone (5) to be chemically similar. Blundell (6) obtained no definite protection against group A streptococcus infections in mice with crude bovine testicular hyaluronidase but observed a greater mean survival time; and McClean (7), using enzyme from a similar source, failed to obtain protection in mice infected with either group A or group C streptococci.

However, Kass and Seastone (8), who recently succeeded in protecting mice to some extent against infection with group A streptococci by using bovine testicular hyaluronidase, ascribed the failure of earlier workers to the use of insufficient amounts of enzyme and to too long an interval between injections. Both of these factors tended to permit the streptococci to regenerate their capsules in the infected host.

In view of these conflicting reports as to the protective effect of hyaluronidase, it seemed desirable to reinvestigate this problem and to attempt to determine the relative significance of the hyaluronic acid capsule and the M protein in the virulence of group A streptococci.

For this purpose, 5 strains of group A streptococci were selected, each of which was available in a glossy and matt form. The glossy variants were mouse-avirulent and produced little or no M protein; the matt variants were made mouse-virulent by animal passage and elaborated large amounts of the M substance. Both variants of each of the 5 strains were morphologically well encapsulated.

As criteria of virulence, two different and distinct systems were employed: (1) an *in vitro* test involving the capacity of the streptococcal cells to resist phagocytosis by leukocytes in human blood; and (2) an *in vivo* test involving the ability of group A streptococci to kill mice following intraperitoneal inoculation. The protective effect of anti-M serum and an enzyme, hyaluronidase, which hydrolyzes the capsular material, was also studied in the *in vivo* system against group A streptococcal infections in mice.

Materials and Methods

Strains of Hemolytic Streptococci.—The 5 strains of group A streptococci were selected because each represented a different serological type, each had a glossy and a matt variant, and both variants were capable of producing good capsules under appropriate conditions.

Griffith's (9) original type strains were used for types 1, 3, and 19. They were designated by him as strains SF 130/2, Lewis opaque 47T, and SF 73/4 and catalogued in this laboratory as T1, T3, and T19 respectively. Strain NY5 was isolated by Stevens and Dochez (10) and used by Griffith as the representative type 10, now designated type 12. Strain S23, type 14, was isolated from the throat of a patient with lobar pneumonia (11). The group C strain, designated D181 in this laboratory, was isolated by Seastone (5) from a guinea pig with chronic lymphadenitis and referred to as strain 4.

Media for Preparation of Cultures.—Todd-Hewitt filtered broth was the most favorable of several media tried for production of well formed capsules (2). Well encapsulated streptococci in the logarithmic phase of growth were prepared by adding to 9 parts of this medium one part of an actively growing 12 hour culture and incubating in a water bath at 37°C. for 3 to 4 hours. Large capsules (Figs. 1-10) were demonstrable by the moist India ink method (12) in both variants of all the strains studied. The cultures were standardized turbidimetrically at a wave length of 520 m μ before each experiment, and this was checked by pouring rabbit blood agar plates to estimate the bacterial population.

The methods of preparing the antisera and M extracts (13, 14), as well as the techniques used in the precipitin and bacteriostatic tests (15, 16), have previously been described. The method for preparing crude bovine testicular extract containing hyaluronidase was that of Kass and Seastone (8). For the mouse virulence and protection experiments, mice of the Rockefeller Institute strain, weighing 15 to 20 gm., were employed.

EXPERIMENTAL

The Relationship between the Production of M Protein and Mouse Virulence of Group A Streptococci.—Experiments were devised to determine the comparative capacities of the equally well encapsulated glossy and matt variants to produce the M substance and to kill mice. From 45 cc. of actively growing 3 hour broth cultures of the encapsulated variants, after a sample was removed for the mouse virulence tests, crude M extracts were prepared. Twofold serial dilutions of these extracts were tested by the capillary precipitin method (15) with homologous, absorbed anti-M serum, as recorded in Table I. A

distinct difference in the amount of the M substance elaborated by the glossy and matt variants of each pair of strains is apparent. Dilutions as high as 1:32 of the extracts of the matt variants showed positive reactions, whereas the undiluted extracts of the glossy variants gave only weak or no precipitin reactions.

For the mouse virulence tests, tenfold serial dilutions were prepared from samples removed from the cultures tested for M substance. The results of these tests are summarized in Table II. A marked difference in mouse virulence between the glossy and matt strains was found in spite of the fact

TABLE I

*Production of Type-Specific M Antigen by Encapsulated Variants of Group A Streptococci
Precipitin Reactions with M Extracts and Homologous Antisera*

Variant.....	Glossy					Matt				
Strain.....	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type.....	1	3	12	14	19	1	3	12	14	19
Titration of M extract										
1:1	±	±	±	0	±	+++	+++±	+++	+++	+++
1:2	0	0	±	0	0	+++	++	+++	+++	+++
1:4	0	0	0	0	0	++	++	+++	++±	+++
1:8	0	0	0	0	0	++	++	+++	++±	+++
1:16	0	0	0	0	0	++	±±	+++±	+++±	++
1:32	0	0	0	0	0	+	±	±	+	+
1:64	0	0	0	0	0	0	0	0	0	0

In titration of M antigen, degree of precipitation is indicated by a ++++ to ± scale; 0 represents no precipitation.

that each variant elaborated equally good hyaluronic acid capsules. All of the encapsulated glossy variants were relatively mouse-avirulent; the encapsulated matt variants were highly virulent, as indicated by death in 3 to 4 days occurring in mice infected with 10^{-8} cc. of culture, containing 2 to 3 microorganisms. Examination of selected mice that died all revealed encapsulated streptococci in the peritoneal exudate or heart blood. In additional experiments it was shown that polymorphonuclear neutrophils and monocytes in the exudate, obtained from the peritoneal cavity of mice inoculated with the encapsulated glossy variants, were actively phagocytizing the streptococci, but only rarely were the encapsulated matt variants taken up by the phagocytic leukocytes.

While these experiments clearly demonstrate the relationship of the M protein to mouse virulence, they do not bring out any evidence that the hyaluronic acid capsule has an effect in enhancing the virulence of these

TABLE II
Mouse Virulence of Encapsulated Variants of Group A Streptococci

Variant	Gloay					Matt				
Strain.....	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type.....	1	3	12	14	19	1	3	12	14	19
Dose cc.										
10 ⁻¹	D1	D1	D1	D1	D1	D1	D1	D1	D1	D1
	D1	D1	D2	D1	D1	D1	D1	D1	D1	D1
	D2	D1	D2	D2	D1	D1	D1	D1	D1	D1
10 ⁻²	S	D2	S	D1	D1	D1	D1	D1	D1	D1
	S	D2	S	D2	D1	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
10 ⁻³	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
10 ⁻⁴	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D2
	S	S	S	S	S	D1	D1	D1	D1	D2
10 ⁻⁵	S	S	S	S	S	D1	D1	D2	D1	D2
	S	S	S	S	S	D1	D1	D2	D2	D2
	S	S	S	S	S	D1	D1	D2	D2	D2
10 ⁻⁶	S	S	S	S	S	D2	D2	D2	D2	D2
	S	S	S	S	S	D2	D2	D2	D2	D3
	S	S	S	S	S	D3	D2	D2	D2	D3
10 ⁻⁷	S	S	S	S	S	D2	D2	D2	D2	D3
	S	S	S	S	S	D3	D2	D2	D3	D3
	S	S	S	S	S	D3	D3	D3	D2	S
10 ⁻⁸	S	S	S	S	S	D3	D3	D3	D3	D3
	S	S	S	S	S	D3	D4	D3	D3	D3
	S	S	S	S	S	D3	D4	D3	S	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

streptococci for mice. Subsequent studies, however, with special techniques, revealed that the capsular material does have some influence on the pathogenicity of these bacteria.

Antiphagocytic Effect of the M Substance and Hyaluronic Acid Capsule.
 In order to analyze the method of destruction of these streptococci in the

animal body, an *in vitro* test was employed, which involved phagocytosis of the streptococci by the leukocytes of normal human blood. Experiments with mouse and human blood revealed that the streptococci were phagocytosed in the blood of both species. Although it was theoretically preferable to use mouse blood as a test system since mouse virulence was being studied, it was found necessary to employ human blood because of the difficulty of obtaining sufficient quantities of blood from mice. Furthermore, the use of human blood was in itself of interest because of the relationship of the virulence of group A streptococci to human infection. Therefore, normal children's blood, which contains no detectable natural opsonins for these microorganisms, was employed.

The crude bovine testicular extract used in all the experiments as a source of hyaluronidase was found, in agreement with earlier workers, not to impair the phagocytic function of the leukocytes. Moreover, this enzyme prevents the formation of capsules of group A and group C streptococci during their growth cycle (Figs. 11 and 12). The question arose as to whether this crude tissue extract might contain proteolytic enzyme capable of digesting the M antigen of the streptococcal cell. Experiments were devised to test this possibility.

Washed group A streptococcal cells, strain S23, matt, type 14, from 500 cc. of Todd-Hewitt broth culture, were suspended in 4 cc. of physiological saline and heat-killed at 56°C. for 30 minutes. To one-half of these cells was added 2 cc. of crude bovine testicular extract containing 200 viscosity-reducing units of hyaluronidase; to the other half was added the same amount of extract inactivated by heating for 30 minutes at 60°C. After incubation of these mixtures at 37°C. for 18 hours, M extracts were prepared, diluted in a twofold serial manner, and tested against homologous antiserum.

Each test sample revealed approximately the same amount of M substance on titration. The same experiment was repeated with the addition to the testicular extract of a reducing substance, thioglycollic acid, in a final concentration of 0.01 M, in order to activate any previously inactive proteolytic enzyme which might be present. Again the M titres obtained were essentially the same in the two systems. In an additional experiment, using M substance in solution, samples of M extract were mixed with an equal volume of testicular extract containing 20 viscosity-reducing units of hyaluronidase or with heat-inactivated testicular extract, and incubated at 37°C. for 18 hours. Twofold serial dilutions of the mixtures were made and tested against homologous antiserum. No evidence of digestion of M substance was obtained. Further studies also revealed that the crude bovine testicular extract failed to digest gelatin or casein. Since the testicular hyaluronidase employed did not digest the M protein on the streptococcal cell or in solution, this extract was considered suitable as a source of hyaluronidase free of proteolytic enzyme.

To determine the antiphagocytic effect of the M substance and hyaluronic acid capsule, opsonic index experiments were performed.

Suspensions of well encapsulated, 3 hour cultures of the glossy and matt variants were turbidimetrically standardized, and blood agar plates were poured to estimate the bacterial count. To each of a series of tubes were added 0.05 cc. of culture dilution containing approximately 1,000,000 streptococci, 0.25 cc. of fresh, heparinized, human blood, and 0.05 cc. of hyaluronidase (10 viscosity-reducing units), type-specific anti-M serum, or saline. After 20 minutes in a rotating machine at 37°C., the tubes were plunged into ice water to stop phagocytosis; and smears were made and stained with Wright-Giemsa solution. The per cent of active leukocytes and the number of cocci ingested by 100 polymorphonuclear neutrophils were determined.

TABLE III

Phagocytic Action of Normal Human Blood on Capsulated and Decapsulated Variants of Group A Streptococci

Variant.....	Glossy					Matt									
Strain.....	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type.....	1	3	12	14	19	1	3	12	14	19	1	3	12	14	19
Antibody.....	None					None					Anti-M Serum				

Capsulated streptococci															
No. of cocci phagocyted by 100 leukocytes.....	1596	1920	1664	1560	2060	39	26	45	46	40	1210	1560	1310	1240	1700
Percentage of active leukocytes.....	97	96	92	84	94	6	2	6	6	10	93	90	94	90	89

Streptococci decapsulated with hyaluronidase															
No. of cocci phagocyted by 100 leukocytes.....	2767	2355	2242	2210	2646	158	210	210	270	315	2340	2410	1780	1700	2710
Percentage of active leukocytes.....	99	93	93	92	96	12	24	20	12	25	95	97	95	91	93

The results of the foregoing experiment are illustrated in Table III. The antiphagocytic properties of the M substance are shown by the fact that the matt, M-producing variants of both capsulated and decapsulated streptococci are markedly more resistant to phagocytosis than the glossy non-M-producing variants. On addition of anti-M serum the encapsulated matt variants become as susceptible to phagocytosis as the encapsulated glossy variants. It will be noted that when hyaluronidase is added to the systems, with resulting removal of the capsules, more of the streptococci are phagocyted than in comparable systems in which the streptococci remain encapsulated because no hyaluronidase is added. These observations suggest that the hyaluronic acid capsule has some antiphagocytic property.

Photomicrographs of representative fields of smears made in the phagocytic experiments are shown for the strain S23, type 14 system. It should be noted that no phagocytosis occurs if M protein is synthesized by the streptococci regardless of whether the capsule is present (Figs. 13 and 14); however, it can be seen (Figs. 15 and 16) that phagocytosis of encapsulated, non-M-containing, glossy variants does take place, but that the decapsulated glossy variants are phagocytosed to a somewhat greater extent. As can be noted in Fig. 15, after phagocytosis of encapsulated organisms the capsule is no longer visible, which suggested that the leukocytes may contain hyaluronidase capable of digesting the capsular material. However, efforts to isolate this enzyme from large amounts of leukocytes obtained from human blood were unsuccessful.

Because the ultimate fate of the streptococci ingested by the phagocytes is not shown by opsonic index experiments such as that just described, a bacteriostatic test was employed. By this method it has been demonstrated that the ingested bacterial cells are destroyed by the phagocytic leukocytes (16).

Tenfold serial dilutions were used of a 3 hour broth culture of well encapsulated streptococci, ranging from 10^{-1} through 10^{-6} . In these experiments, 200 to 300 bacterial cells were contained in the 10^{-6} dilutions. To each of a series of tubes were added 0.05 cc. of culture dilution, 0.05 cc. of hyaluronidase (10 viscosity-reducing units), saline, or type-specific anti-M serum, and 0.25 cc. of fresh, heparinized, human blood. After incubation at 37°C . in a rotator for 3 hours, samples from each mixture were removed and streaked on rabbit blood agar plates. The resulting growth after 18 to 24 hours' incubation at 37°C . was recorded on a ++++ to 0 scale.

Typical experiments are shown in Table IV, which reveals that the encapsulated, glossy variants are extremely susceptible to phagocytosis and fail to survive in the blood; on the other hand, the matt variants are resistant. In the presence of homologous anti-M serum these matt variants become as susceptible as the glossy variants. When hyaluronidase is added, which keeps the streptococci from regenerating their capsules throughout the 3 hour experimental period, it can be noted that the decapsulated matt variants retain their resistance to the bacteriostatic action of the blood. The decapsulated as well as the encapsulated matt variants become as susceptible on inclusion of anti-M serum in the system as the glossy variants.

It appears from the findings of these *in vitro* experiments that the M substance is far more important than the hyaluronic acid capsule in causing the streptococci to resist the phagocytic and bacteriostatic effect of the blood, but there was a definite indication from the opsonic index experiments that removal of the capsule enhanced the phagocytic effect of the leukocytes.

Demonstration of Protective Capacity of Crude Bovine Testicular Hyaluronidase against Group C Streptococcal Infection in Mice.—Although the rôle played by

TABLE IV
Bacteriostatic Action of Normal Human Blood on Capsulated and Decapsulated Variants of Group A Streptococci

Variant.....	Glossy					Matt				
	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
	1	3	12	14	19	1	3	12	14	19
Serological type.	None					None				
Antibody.....	Anti-M serum									
Capsulated streptococci										
Dilution of culture										
10 ⁻¹	++++	++++	+++	+++	+++	++++	++++	+++	+++	+++
10 ⁻²	++±	++±	+	+	++	++±	++±	++	++	++
10 ⁻³	+	7	0	+	++	+	4	6	1	+
10 ⁻⁴	9	0	0	0	3	2	2	4	0	6
10 ⁻⁵	0	0	0	0	0	0	0	0	0	1
10 ⁻⁶	0	0	0	0	0	0	0	0	0	0
Streptococci decapsulated with hyaluronidase										
Dilution of culture										
10 ⁻¹	++++	++++	+++	+++	+++	++++	++++	+++	+++	+++
10 ⁻²	++	+	9	9	++±	++	++	+	++	++
10 ⁻³	+	2	0	0	+	+	0	7	1	+
10 ⁻⁴	0	0	0	0	0	0	0	0	0	7
10 ⁻⁵	0	0	0	0	0	0	0	0	0	0
10 ⁻⁶	0	0	0	0	0	0	0	0	0	0

In bacteriostasis of strains, degree of growth is indicated on a + to + scale; fewer than 10 colonies are represented in arabic numerals; 0 indicates no growth.

the M protein of the streptococcal cell in resisting the phagocytic action of leukocytes contained in human blood appeared clearly defined in the *in vitro* experiments, the effect of the capsule was still not well understood. We, therefore, attempted to study the problem in an *in vivo* system employing the mouse as a test animal.

To make certain that the preparation containing hyaluronidase was active in the *in vivo* system selected, the bovine testicular extract was tested for its effect in protecting mice against infection with group C streptococci. Previous workers, with one exception, obtained good protection in similar experiments.

The procedure employed was essentially the same as that described by Kass and Seastone (8) for protection experiments with group A streptococci. Mice were inoculated intraperitoneally with tenfold serial dilutions of 4 hour broth cultures of encapsulated group C streptococci, strain D181. The number of streptococci inoculated were calculated by pouring rabbit blood agar plates and the colony counts were 26 in the 10^{-7} dilution and 240 in the 10^{-6} dilution. Treatment with 0.5 cc. crude bovine testicular extract containing 100 viscosity-reducing units was started 2 hours later. Injections of enzyme were given intraperitoneally every 2 hours for the first 12 hours, every 4 hours during the next 36 hours, and every 12 hours for the last 48 hours; a total of 19 injections.

In Table V are illustrated the results of the protective influence of crude bovine testicular hyaluronidase on mice infected with group C streptococci. The enzyme protected all mice completely against 1,000 M.L.D. and some of the mice against even 100,000 M.L.D. A control group of mice treated with enzyme inactivated by heating to 60°C. for 30 minutes died as rapidly as the untreated virulence controls. These findings are remarkably similar to those of Hirst (4), who employed leech extract as a source of enzyme.

Method of Demonstrating Mouse Protection against Group A Streptococci with Hyaluronidase.—Since crude testicular hyaluronidase was thus found to be effective in protecting mice against infections with encapsulated group C streptococci (Table V), experiments were undertaken in an attempt to also obtain protection in mice with encapsulated group A streptococci. In the studies of Kass and Seastone (8), who were successful in such experiments, it was noted that the final dilutions of the streptococci were made in testicular extract and allowed to remain for 5 minutes at room temperature before injection of the mixture; streptococci used for the controls, however, were diluted in plain broth. Blundell (6) had previously shown that simultaneous injections of a mixture of bovine testicular extract and streptococci, followed by treatment with the enzyme, provided the greatest delay in the time of death as compared with untreated control mice. These observations suggested an explanation for the fact that we had earlier failed to obtain any protection of mice against group A streptococci since 2 hours had elapsed before enzyme treatment was started.

The effect of giving the hyaluronidase simultaneously with the streptococci

by diluting the bacteria in the testicular extract was compared with the effect of delaying treatment by diluting the streptococci in plain broth and giving the first injection of enzyme 2 hours later. The results of such an experiment are shown in Table VI. Encapsulated group A type 14 streptococci, strain S23, were employed. The colony count in the 10^{-7} dilution was 19 colonies and in the 10^{-8} dilution, 2 colonies. The treatment schedule was the same as that used against group C streptococci, except where earlier treatment was

TABLE V
Demonstration of Protective Activity of Crude Testicular Hyaluronidase
Mouse Protection against Group C Streptococci

Treatment of mice	Culture of streptococci, strain D181					
	cc.					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Unheated testicular hyaluronidase	D1	D2	S	S	S	S
	D2	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
Heated testicular hyaluronidase*			D1	D1	D1	D1
			D1	D1	D1	D2
			D1	D1	D2	D2
			D1	D2	D2	D2
Untreated virulence controls	D1	D1	D1	D1	D1	D1
	D1	D1	D1	D1	D1	D2
	D1	D1	D1	D2	D2	D2
	D1	D1	D1	D2	D2	D2

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

* Hyaluronidase heated at 60°C. for 30 minutes.

given by diluting the cultures in hyaluronidase. In diluting the streptococci in hyaluronidase, the concentration of enzyme was adjusted so that the mouse received the same amount of enzyme as that used in the later therapeutic doses; i.e., 100 viscosity-reducing units.

When the streptococci were diluted in plain broth and the mice subsequently treated after a lapse of 2 hours with 19 injections of hyaluronidase for 96 hours, only a slight delay in the rate of death was obtained as compared with the untreated controls. In contrast, when the streptococci were diluted in a solution of hyaluronidase, even though no additional treatment was given, there was some protection in the 10^{-8} dilution and a delay in the time of death in the other dilutions. Moreover, when the mice, in addition to the first dose

TABLE VI

Method of Demonstrating Protective Action of Crude Testicular Hyaluronidase against Group A Streptococci
Mouse Protection Test

Treatment of mice		Culture of streptococci, strain S23 matt, type 14				
		cc.				
		10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Streptococci diluted in plain broth	Untreated virulence controls	D1	D1	D1	D2	D3
		D1	D1	D1	D2	D3
		D1	D1	D1	D2	D3
		D1	D1	D2	D2	D3
		D1	D1	D2	D2	D3
		D1	D2	D2	D3	D4
	Treated with multiple injections of hyaluronidase	D1	D1	D2	D3	D3
		D1	D1	D2	D3	D3
		D1	D1	D2	D3	D4
		D1	D2	D3	D3	D4
		D2	D2	D3	D3	D4
		D2	D2	D4	D4	S
Streptococci diluted in solution of hyaluronidase	No additional treatment	D1	D2	D3	D4	D5
		D1	D2	D3	D4	D5
		D2	D2	D3	D4	D5
		D2	D2	D3	D5	S
		D2	D2	D4	D5	S
		D2	D2	D4	D5	S
	Treated with multiple injections of hyaluronidase	D1	D1	D4	D5	S
		D1	D2	D5	S	S
		D1	D3	D5	S	S
		D2	D3	D6	S	S
		D2	D3	D5	S	S
		D2	D3	S	S	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

Duplicate sets of mice treated with hyaluronidase heated at 60°C. for 30 minutes all died at the same rate as untreated virulence controls.

of hyaluronidase, were also treated with multiple injections (19 injections for 96 hours), protection was obtained against 10 M.L.D., with marked delay in the time of death of the other mice, as compared with the untreated virulence controls.

It is thus apparent that protection against group A streptococcal infection in mice can be obtained when hyaluronidase is given concomitantly with the

TABLE VII

Combined Action of Antiserum and Crude Testicular Hyaluronidase in Protecting Mice against Infection with Group A Streptococci
Mouse Protection Tests

Treatment of mice		Culture of streptococci, strain S23 matt, type 14							
		cc.							
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Antiserum alone	Single injection of antiserum given 16 hrs. before streptococci	D1	D3	S	S	S	S	S	S
		D1	D5	S	S	S	S	S	S
		D1	D6	S	S	S	S	S	S
		D2	S	S	S	S	S	S	S
		D3	S	S	S	S	S	S	S
		D3	S	S	S	S	S	S	S
Testicular hyaluronidase alone	Multiple injections of hyaluronidase*					D3	D3	D7	S
						D3	D3	S	S
						D3	D3	S	S
						D3	D3	S	S
						D3	D3	S	S
Both antiserum and testicular hyaluronidase	Each given as above*	D2	S	S	S	S			
		D3	S	S	S	S			
		D3	S	S	S	S			
		D3	S	S	S	S			
		S	S	S	S	S			
		S	S	S	S	S			
Control: antiserum and saline	Antiserum as above: multiple injections of saline instead of hyaluronidase	D1	D2	S	S				
		D1	D5	S	S				
		D1	S	S	S				
		D2	S	S	S				
		D2	S	S	S				
		D2	S	S	S				
Untreated virulence controls		D1	D1	D1	D1	D2	D2	D2	
		D1	D1	D1	D1	D2	D2	D4	
		D1	D1	D1	D2	D2	D2	D4	
		D1	D1	D1	D2	D2	D2	D4	
		D1	D1	D2	D2	D2	D2	D5	
		D1	D1	D2	D2	D2	D4	S	

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

* Streptococci used for inoculation of these mice were diluted in hyaluronidase.

streptococci and followed by additional treatment with the enzyme, but not if the streptococci are diluted in broth and injected into mice, followed 2 hours later by treatment with hyaluronidase.

Combined Effect of Anti-M Serum and Hyaluronidase in Mouse Protection against Group A Streptococci.—Having demonstrated a method by which some protection of mice against encapsulated group A streptococci can be obtained by using hyaluronidase, thus confirming the work of Kass and Seastone (8), the protective effect of type-specific anti-M serum was compared with that of hyaluronidase; and the combined effect of these 2 agents was also investigated.

The method of treatment, which was demonstrated as being most effective in the preceding experiment (Table VI), was used. Five groups of mice were selected and each treated in a different manner. The first group was injected intraperitoneally with 0.5 cc. type-specific antiserum 16 hours prior to intraperitoneal inoculation with the streptococci, and no further treatment was given. The second group was inoculated with streptococci diluted in hyaluronidase and these mice were further treated with multiple injections of this substance as in the preceding experiment (Table VI). The third group received the combined treatment of the first two groups; *i.e.*, the mice received antiserum, were inoculated with streptococci diluted in hyaluronidase, and were further treated with multiple injections of the enzyme. The fourth group, used as a control for the effect of repeated injections, was treated with antiserum as in the first group, and received multiple injections of saline instead of hyaluronidase after inoculation of streptococci. The fifth group served as untreated virulence controls.

Table VII summarizes the findings in this experiment. With a single injection of antiserum, protection against 10,000 to 100,000 M.L.D. of group A streptococci was obtained. Protection against 10 M.L.D. was noted with hyaluronidase. With the combination of antiserum and testicular hyaluronidase there was an additive effect affording protection against 1,000,000 M.L.D., as compared with the untreated virulence controls. The control group, given antiserum and multiple injections of saline instead of hyaluronidase, revealed that there was no non-specific protective effect from the multiple injections.

The results of this experiment show that hyaluronidase, effective against the capsular material, and type-specific anti-M serum, specifically directed against the M substance of the streptococcal cell, each has a protective effect against group A streptococcal infection in mice. These findings also illustrate that the anti-M serum protects against 1,000 to 10,000 times as many M.L.D. as hyaluronidase and that the combined action of these two substances provides greater protection for mice than either one alone.

DISCUSSION

The protective effect of hyaluronidase derived from various sources has usually been unquestioned with regard to experimental group C streptococcal infections in laboratory animals, but conflicting evidence has been obtained by different investigators as to its protective action in group A streptococcal infections in mice. This has led to different concepts as to the influence of the hyaluronic acid capsule on the virulence of group A streptococci. Those workers, notably Hirst (4), who failed to protect mice against group A streptococcal infections with hyaluronidase, concluded from their studies that,

unlike group C streptococci, other factors such as the M antigen were more significant than the capsule in determining the virulence and invasiveness of group A streptococci.

The present work provides an explanation for the discrepancy between this point of view and that of Kass and Seastone (8) who, on finding some protection against group A streptococcal infection in mice treated intensively with hyaluronidase, expressed the view that the capsules of both group A and group C streptococci are responsible for the greater part of their virulence. Furthermore, these workers believed that the M antigen might be of only minor importance, since group A streptococci may elaborate M substance without necessarily being virulent for mice.

The evidence obtained in this study brings out the fact, well recognized by all investigators, that no single cellular component or product of group A streptococci, or indeed probably of any microorganism, contributes exclusively to the property of virulence. It is also recognized that factors still not known are essential to the development of virulence for different animal species. As an illustration, M-containing, encapsulated variants freshly isolated from patients, although evidently virulent for man are usually avirulent for mice. Apparently other cellular functions in addition to the production of capsular polysaccharide or M protein are also involved in the virulence of these microorganisms for mice. However, unless the strain elaborates the M antigen, it is not virulent for either mouse or man. Of the known factors, both the hyaluronic acid capsule and the M antigen of the streptococcal cell have been shown to contribute to the exhibition of virulence, and correspondingly the agents which lead to their destruction contribute to the protection of mice against infections with these streptococci. Thus, it has been amply demonstrated that the matt variants which elaborate the M substance are virulent for mice and resist phagocytosis; the glossy variants, however, which produce little or no M substance, are avirulent for mice and are susceptible to phagocytosis, even though both variants are encapsulated. This offers substantial evidence that the M substance is an important factor in the pathogenicity of group A streptococci.

In phagocytic experiments, on comparing the encapsulated streptococci and streptococci decapsulated with hyaluronidase, it was noted that the decapsulated bacteria were taken up by the leukocytes more readily and in larger numbers than the encapsulated forms. Further evidence was provided that the capsule is actually concerned with virulence to a certain extent by demonstrating that mice were protected against 10 M.L.D. of group A streptococci by hyaluronidase treatment.

To obtain this protective effect, it was essential to have the capsules removed by diluting the streptococci in a solution of hyaluronidase before injection and further to prevent capsule regeneration by frequent and continued

treatment with enzyme. No protection was obtained if the streptococci were diluted in broth and the multiple injections of hyaluronidase started 2 hours later, because the injected streptococci had multiplied so many times during the 2 hour interval that the enzyme therapy was inadequate to permit the host to deal with the increased number of bacteria. The technique of simultaneous injection of enzyme and streptococci explained the success of Kass and Seastone (8) in obtaining protection in mice; and it is likely that other workers also would have shown protective effects if such early and intensive treatment had been carried out.

The protection of mice could be increased from the 10 M.L.D. obtained by enzyme therapy alone to 1,000,000 M.L.D. by the combined use of anti-M serum and enzyme. A single injection of 0.5 cc. of antiserum 16 hours before inoculation of mice was sufficient to afford almost this much protection; and previous work indicates that even this dose of serum was a great excess (17). The greater protection afforded by the anti-M serum emphasizes the importance of the M substance in virulence of group A streptococci and is in agreement with other studies in this report dealing with the opsonic index, bacteriostatic, and mouse virulence experiments.

As previously demonstrated by Ward and Lyons (18) and shown in this report, encapsulation does not necessarily exclude phagocytosis. In the present experiments, decapsulated streptococci were slightly more susceptible to phagocytosis than those which retained their capsules. The encapsulated variants were nevertheless still highly susceptible to phagocytosis unless in addition to capsular substance they elaborated M antigen, whereas M-producing variants were only phagocytosed to a very slight extent even though they were decapsulated with hyaluronidase. This suggests that encapsulation of group A streptococci and resistance to phagocytosis do not bear a direct relation to each other.

Though it is hazardous to generalize from these experiments in mice that the M substance and the capsular material play similar rôles in infections in man, the natural host for group A streptococci, it is worthy of note that one test system employed was dependent upon the phagocytic leukocytes in normal human blood. Both the M substance and the capsules had anti-phagocytic properties against these human leukocytes. That the M protein is intimately related to virulence of streptococci for man is further supported by the fact that in this laboratory no strain tested and shown to lack M antigen has ever been isolated from a patient in the acute phase of streptococcal infection. In all strains tested, whenever the M substance could not be identified with the available type-specific antisera, it was possible by immunizing rabbits to show that the strain produced some other M substance not previously identified in this laboratory. On the other hand, strains have been isolated from patients in the acute phase of their infection which lacked

the capacity to produce the capsular polysaccharide; this is most strikingly illustrated in the case of strains of serological types 4 and 22 which produce hyaluronidase (19) and therefore do not develop capsules.

On the basis of the evidence presented, it appears that the hyaluronic acid capsule is one of the factors influencing virulence of group A streptococci, but that the M antigen appears to be a far more important factor in determining this property.

SUMMARY

Five strains of encapsulated group A streptococci of different serological types, each with a glossy and a matt variant, were studied to compare the rôles of the M substance and the hyaluronic acid capsule in virulence of these microorganisms. The results indicated that both contribute to the virulence of group A streptococci but that the M antigen is the more fundamental factor.

Encapsulated variants, both glossy and matt, were slightly less susceptible to phagocytosis than those from which the capsule had been removed with hyaluronidase. Glossy variants, containing no M substance, were readily phagocytized; matt, M-containing variants were resistant to phagocytosis except in the presence of anti-M serum when they became fully susceptible.

Only the M-containing, matt strains were mouse-virulent. Mice were protected against infections with these strains:

(a) By removal of the capsule with hyaluronidase, which resulted in slight protection, but only against 10 M.L.D. Early and intensive treatment was required to produce this effect; *i.e.*, simultaneous injection of enzyme and streptococci followed by prolonged enzyme therapy.

(b) By a single injection of anti-M serum administered the day before inoculation of the streptococci, which resulted in protection against 100,000 M.L.D.

(c) By combined use of enzyme and anti-M serum, an additive effect of the two protective agents occurred, which resulted in protection against 1,000,000 M.L.D.

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EXPLANATION OF PLATES

These photographs were made by Mr. Joseph B. Haulenbeek.

PLATE 13

All preparations made with India ink; 3½ hour broth cultures of group A streptococci employed. × 1500.

FIG. 1. Capsule of glossy variant of type 1 (strain T1).

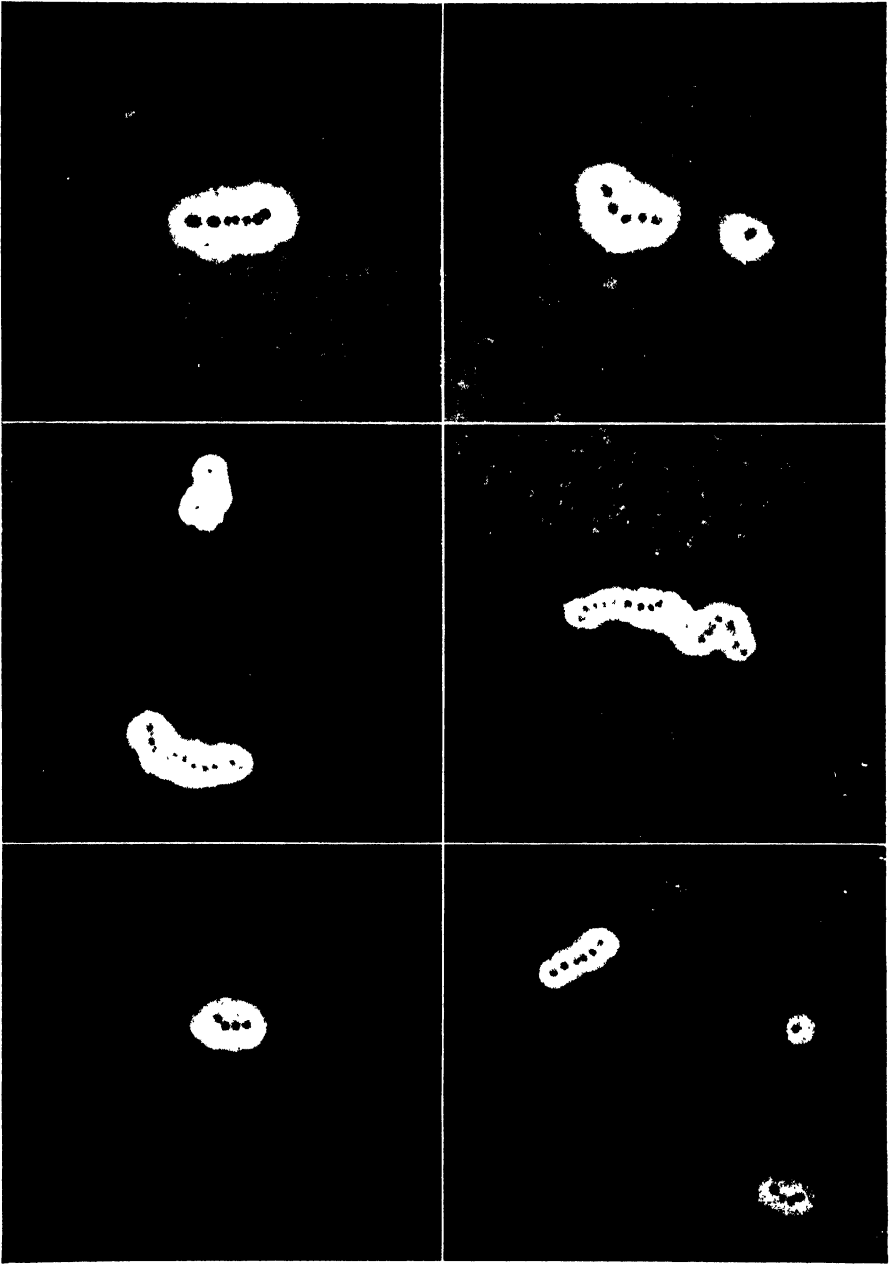
FIG. 2. Capsule of matt variant of type 1 (strain T1).

FIG. 3. Capsule of glossy variant of type 3 (strain T3).

FIG. 4. Capsule of matt variant of type 3 (strain T3).

FIG. 5. Capsule of glossy variant of type 12 (strain NY5).

FIG. 6. Capsule of matt variant of type 12 (strain NY5).



(Bothhard: Group A staphylococcus infections)

PLATE 14

FIG. 7. Capsule of glossy variant of type 14 (strain S23).

FIG. 8. Capsule of matt variant of type 14 (strain S23).

FIG. 9. Capsule of glossy variant of type 19 (strain T19).

FIG. 10. Capsule of matt variant of type 19 (strain T19).

FIG. 11. Decapsulated glossy variant of type 14 (strain S23); 3 minutes after addition of bovine testicular extract.

FIG. 12. Decapsulated matt variant of type 14 (strain S23); 3 minutes after addition of bovine testicular extract.

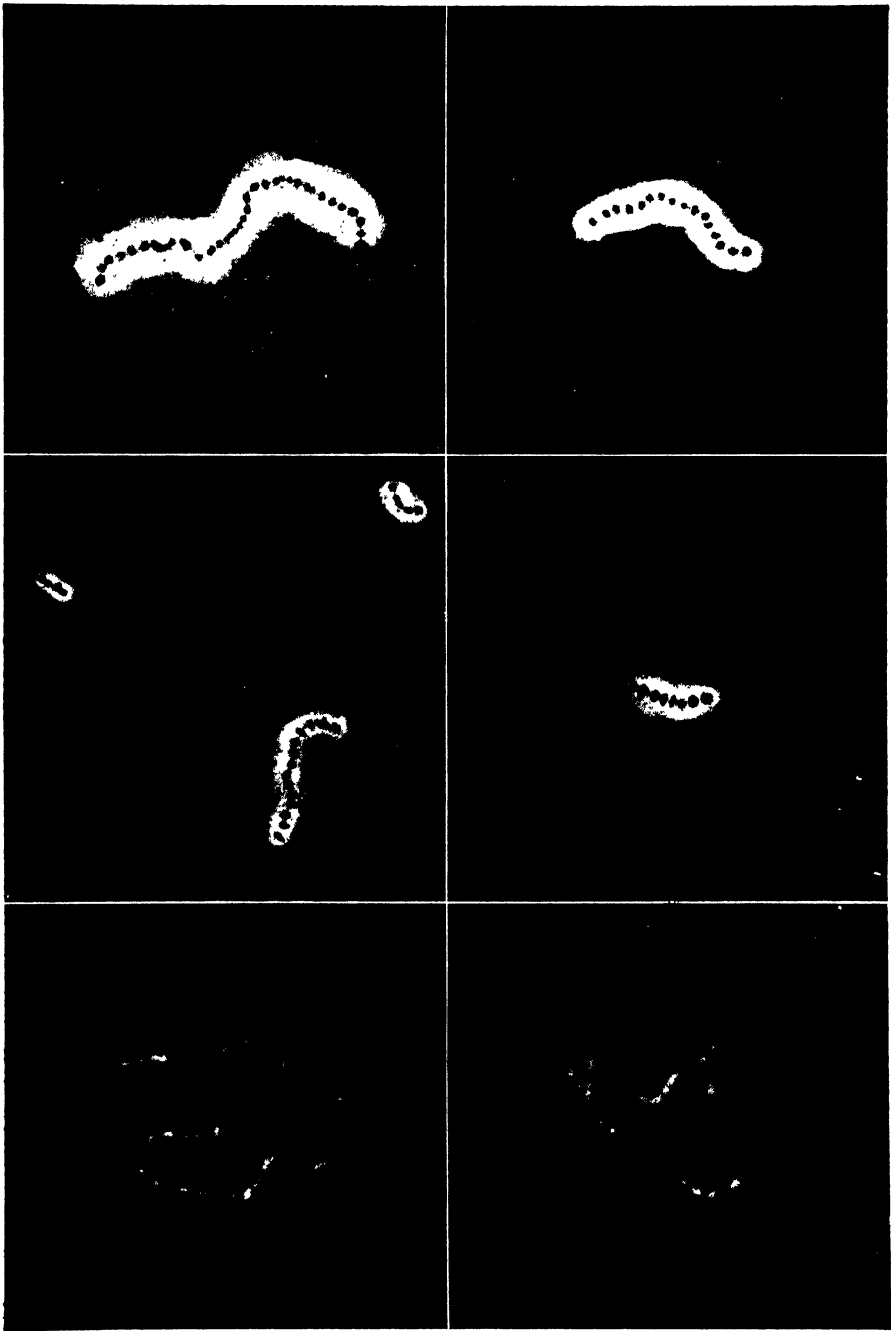


PLATE 15

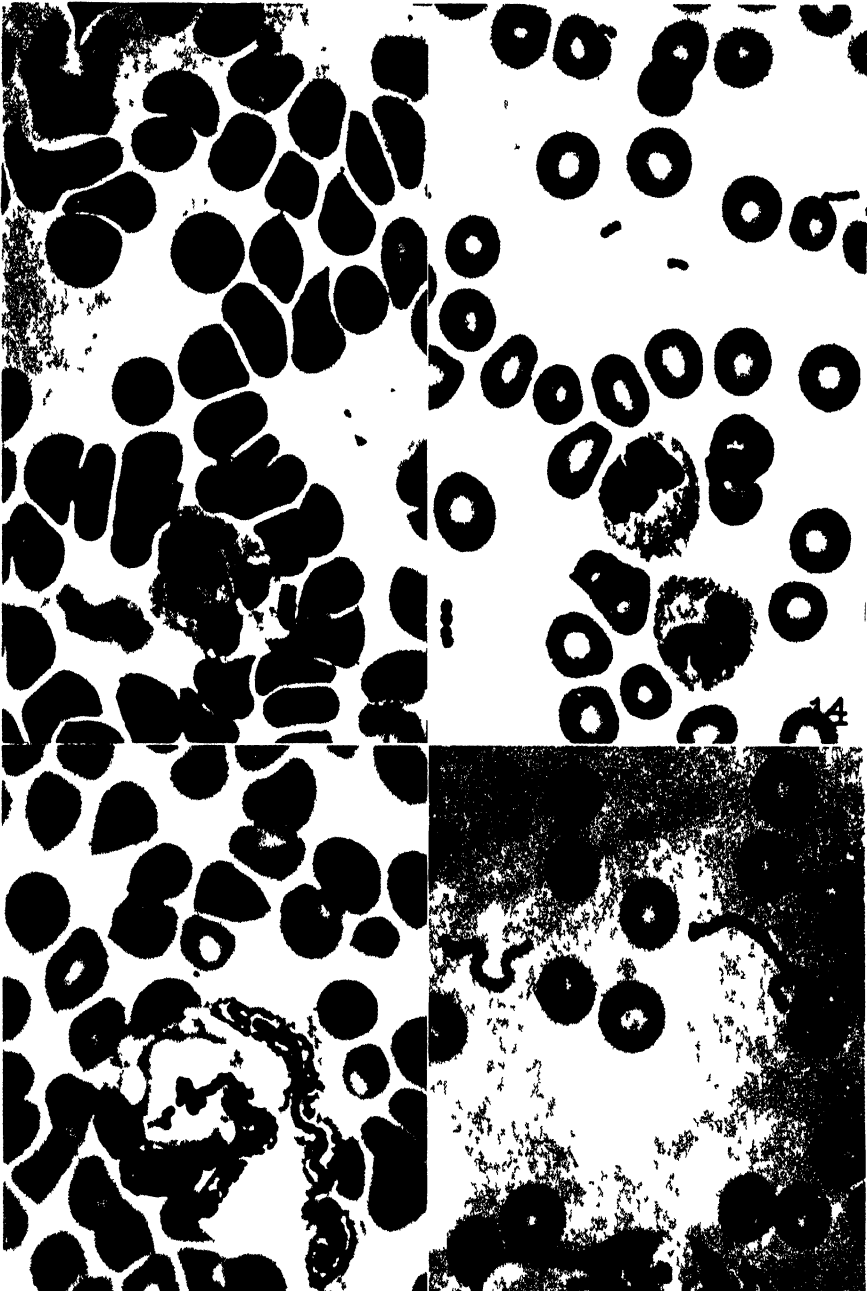
Films stained with Wright and Giemsa solution. These are representative specimens obtained from phagocytic experiment shown in Table III. $\times 1500$.

FIG. 13. Capsulated matt variant of group A streptococci, type 14, (strain S23) not phagocyted by polymorphonuclear leukocyte.

FIG. 14. Decapsulated matt variant of group A streptococci, type 14, (strain S23) also not phagocyted by polymorphonuclear leukocytes. Bovine testicular extract employed in system to remove streptococcus capsule.

FIG. 15. Capsulated glossy variant of group A streptococci, type 14, (strain S23) being phagocyted by polymorphonuclear leukocyte. Note phagocytosis despite presence of capsule.

FIG. 16. Decapsulated glossy variant of group A streptococci, type 14, (strain S23) being phagocyted by polymorphonuclear leukocytes. Bovine testicular extract employed in system to remove streptococcus capsule. Note increased number of decapsulated streptococci being phagocyted as compared with encapsulated streptococci shown in Fig. 15.



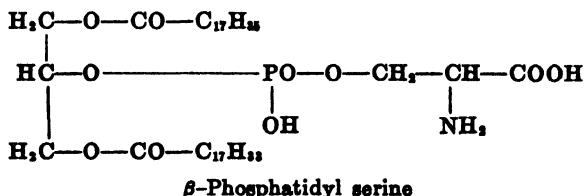
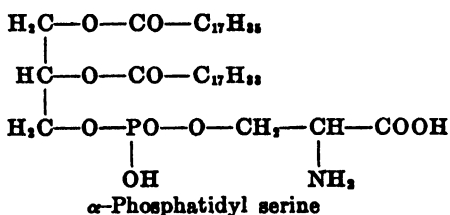
THE CHEMICAL STRUCTURE OF PHOSPHATIDYL SERINE

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Phosphatidyl serine is the name given to a phosphatide which has been isolated from brain (1, 2). This paper reports work according to which the chemical structure of phosphatidyl serine, prepared by the method described, corresponds to one of the two accompanying formulas in which the position of the fatty acids and phosphoryl radicals is arbitrary, since there is as yet no reliable way of ascertaining whether glycerophosphoric acid in phosphatides belongs to the α or the β form (3). The name of phosphatidyl serine was chosen because this compound appears to be an ester of serine and a phosphatidic acid (4).



The postulated formula is based on the following facts: (1) Chemical analyses of phosphatidyl serine (freed of base) for C, H, N, P, carboxyl N, $\text{NH}_2\text{-N}$, and fatty acids agree very closely with the theoretical values calculated from the postulated formula. (2) Glycerophosphoric acid, L-serine, and fatty acids have been isolated as cleavage products of phosphatidyl serine in molecular proportions approximately 1:1:2. (3) Phosphatidyl serine reacts with ninhydrin (5) and with HNO_2 (6) in the same way as an α -amino acid. This shows that both the $-\text{COOH}$ and the $-\text{NH}_2$ groups of serine are free in the intact molecule of phosphatidyl serine.

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On the other hand, phosphatidyl serine does not react with HIO_4 . Since HIO_4 is known to react with compounds with a $-\text{CH}(\text{NH}_2)-\text{CH}(\text{OH})-$ group, and specifically with serine (7), the fact that phosphatidyl serine does not react with HIO_4 is conclusive evidence that either its $-\text{OH}$ or its $-\text{NH}_2$ group is combined. And since its $-\text{NH}_2$ group is shown to be free both by the ninhydrin (5) and the HNO_2 reaction (6), it is obvious that the $-\text{OH}$ group is combined. (4) From the postulated formula, phosphatidyl serine exhibits one free basic group, namely the $-\text{NH}_2$ group, and two acidic groups, namely the $-\text{COOH}$ group of serine and one group from phosphoric acid. This should give a strongly acidic compound that would bind 1 equivalent of base at the physiological pH. That this is the case is shown by the fact that phosphatidyl serine, isolated from brain by the use of neutral solvents and freed of water-soluble impurities by dialysis, contains K and Na, and that the ratio (equivalence of base)/(atoms of P) is 1.00. That these bases are combined with phosphatidyl serine in a salt-like combination is shown by the fact that they can be removed by treatment with 0.05 N HCl, base-free phosphatidyl serine being thereby obtained. From the free acid the original phosphatidyl serine K-Na salt can be prepared by the addition of the amount of base theoretically required.

Serine has been isolated as serine *p*-hydroxyazobenzene-*p*-sulfonate (8). It accounts for 80.7 per cent of the carboxyl N present in the starting material. From it L-serine has been recovered in a yield that accounts for 73.5 per cent of serine present in phosphatidyl serine, according to the postulated formula. The impure barium glycerophosphate obtained accounts for 81 per cent of P in starting material, and the analytically pure barium glycerophosphate for 60 per cent of the original constituent P.

Estimation of fatty acid gives for phosphatidyl serine a (moles of fatty acid)/(atoms of P) ratio of 2.00. The neutral equivalent found for the fatty acids was 283, which is the theoretical value for an equimolar mixture of oleic and stearic acids. By the lead precipitation method, phosphatidyl serine fatty acids were divided equally between saturated and unsaturated acids. From the former, pure stearic acid has been obtained. The amount obtained accounts for 69 per cent of the amount present in phosphatidyl serine, according to the postulated formula. The unsaturated fatty acid fraction appears to be mostly oleic acid, although attempts to obtain this acid in pure form have failed so far.

The iodine number calculated from the postulated formula is 31.5. The iodine number found varied between different preparations, ranging from 33.0 to 40. This discrepancy can be explained, at least in part, by the fact that our preparations were only between 92 and 97 per cent pure (as evaluated by their (carboxyl N ratio)/(total N)) and that the main

contaminant appeared to be phosphatidyl ethanolamine, which has a higher iodine number than phosphatidyl serine (2).

It must be emphasized that this paper deals only with phosphatidyl serine isolated by the method described. It accounts for about 60 per cent of all the lipide carboxyl N from brain. However, the possible existence of phosphatidyl serine having fatty acids other than stearic and oleic acids as constituents must be kept in mind. From another fraction isolated from brain cephalin, to which the name of inositol phosphatide was given for descriptive purposes (2), other preparations of phosphatidyl serine have been isolated, incidental to the isolation of diphosphoinositide (9). These phosphatidyl serine preparations are found to contain Na instead of K as the most abundant inorganic base.

EXPERIMENTAL

Analytical Methods—Manometric methods were routinely used for the estimation of C (10), P (11), N (12), and carboxyl N (5). Carboxyl N analysis on phosphatidyl serine gives better results when 1 cc. of water is added to the weighed dry material and the sample allowed to stand for a few minutes with occasional gentle shaking, so as to allow it to form an emulsion. In the case of base-free phosphatidyl serine, 1 or 2 drops of 0.1 N NaOH are added to facilitate emulsification. 13 minutes in the boiling water bath are allowed for reaction with ninhydrin, this length of time having been found to yield good checks between parallel estimations. In the later stages of work P has been estimated by Sperry's method (13), iodine numbers by Yasuda's method (14), and barium by weighing it as BaSO₄. NH₂-N was estimated manometrically on acid hydrolysates, as described elsewhere (2), to eliminate interference from unsaturated fatty acids (15). K was estimated as potassium phosphotungstate (16), and Na as sodium uranyl zinc acetate by a microgravimetric modification of the method of Salit (17). Ca and Mg were estimated by standard methods (18), modified to suit the type of material dealt with.

In cases in which elementary composition has been used for identification of compounds or in which values are recorded as evidence, C and H were estimated by dry combustion,¹ lead chromate being used in compounds that had base, N by the Dumas method, and P gravimetrically as ammonium phosphomolybdate. It has been found that C values by the wet combustion manometric method of Van Slyke and Folch (10) agreed with those obtained by dry combustion.

Glycerol was estimated by the Blix method (19), which has been found to give recoveries of only 95 to 96 per cent when tested with standard glycerol, or glycerophosphate solutions.

¹ Analyses run by Dr. E. W. Elek.

Preparation and Properties of Phosphatidyl Serine

Phosphatidyl serine is prepared from brain cephalin by the chloroform-alcohol method of fractionation, freed of water-soluble impurities by dialysis, and lyophilized as described elsewhere (2). Usually this method yields preparations of 85 to 90 per cent purity; i.e., between 85 and 90 per cent of the nitrogen present is carboxyl N. When the percentage of total N as carboxyl N is less than the stated percentage, it is easy to bring it up to this level by the following procedure.

1 gm. of the preparation under study is dissolved in 10 cc. of chloroform, and 16.5 cc. of absolute ethyl alcohol are added to the solution. A turbidity develops, and on standing or by centrifugation the system resolves itself into an underlying viscous layer and a clear supernatant solution. The clear supernatant solution is decanted, and to it are added 30 cc. of absolute ethyl alcohol. A precipitate separates which is collected and dried. On analysis it is found to contain carboxyl N at a higher concentration than the mother substance. In a typical case, by this procedure the following fractions have been obtained from 22.6 gm. of a preparation containing 1.25 per cent carboxyl N: (1) recovered from the viscous underlying layer, 7.4 gm. of material containing 1.29 per cent carboxyl N; (2) the precipitate collected from the supernatant solution, 11.7 gm. of material containing 1.43 per cent carboxyl N; (3) recovered from the supernatant solution (2), 3.2 gm. of material containing 0.35 per cent carboxyl N.

We have been unable to obtain consistently phosphatidyl serine having more than 92 per cent of its N as carboxyl N. Occasionally, preparations have been obtained showing concentrations of carboxyl N as high as 97 per cent of the total N. The main contaminant appears to be phosphatidyl ethanolamine, as is shown by the fact that all N present is $\text{NH}_2\text{-N}$. In search of the nature of other contaminants phosphatidyl serine was found to be essentially free of cerebrosides (carbohydrates <0.1 per cent), lecithin, or sphingomyelin (choline <0.1 per cent) and cholesterol (<0.1 per cent).

Phosphatidyl serine is obtained as a loose white powder. On being dissolved in organic solvents and recovered from solution, it acquires a light tan color. After lyophilization, phosphatidyl serine keeps fairly well if stored *in vacuo* in the dark. Under these conditions it retains a certain amount of water. For instance, 401.75 mg. of phosphatidyl serine that has reached constant weight *in vacuo* over CaCl_2 at room temperature show a loss of weight of 6.65 mg. (i.e., 1.65 per cent) on being heated in a vacuum at 80° to constant weight. Restored to former conditions (i.e., in a vacuum at room temperature), it regains its former weight within 48 hours.

Phosphatidyl serine is freely soluble in chloroform, ethyl ether, and petroleum ether, and insoluble in ethyl alcohol, methyl alcohol, or acetone. On standing in contact with water, it forms an emulsion. These emulsions are very stable and an 8 per cent emulsion of phosphatidyl serine will stand centrifugation at 4000 R.P.M. for an indefinite period of time.

Bases Combined with Phosphatidyl Serine—Phosphatidyl serine preparations obtained by the method described are found to contain 1 equivalent of inorganic base for each atom of P. K is by far the most abundant base with a smaller amount of Na, but neither Ca nor Mg is present. Detailed results on this point are given in Table I for the two preparations. Studies carried out on cephalin preparations (20) (presumably containing about

TABLE I
Analysis of Two Preparations of Brain Phosphatidyl Serine

Components	Preparation Oct. III	Calculated for BCaH ₇ O ₁₀ NP*	Preparation Sept. IIIbII	Calculated for BCaH ₇ O ₁₀ NP†
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C	60.8	61.1	61.71	61.50
H	9.32	9.59	9.87	9.60
N	1.65	1.70	1.65	1.71
P	3.75	3.76	3.76	3.78
COOH-N	1.52	1.70	1.57	1.71
Iodine No.	40		38.0	

* B stands for the cations present. Preparation Oct. III contained 3.76 per cent K and 0.51 per cent Na; (equivalence of base)/(atoms of P) = 0.98. The values given in this column have been calculated for oleylstearyl-glycerolphosphoryl serine corrected for the cations present.

† B stands for the cations present. Preparation Sept. IIIbII contained 2.93 per cent of K and 0.99 per cent of Na; (equivalence of base)/(atoms of P) = 0.98. The values given in this column have been calculated for oleylstearyl-glycerolphosphoryl serine corrected for the cations present.

50 per cent phosphatidyl serine (21)) have shown that these bases are combined in un-ionized form.

Analyses of Phosphatidyl Serine—The results of chemical analysis of phosphatidyl serine are given in Table I. It is seen that the results obtained closely agree with values calculated for the postulated formula corrected in each case for the amount of inorganic bases present in the preparation.

Preparation of Base-Free Phosphatidyl Serine

The ability of phosphatidyl serine to form fairly concentrated emulsions in water depends upon the presence of K or Na combined in its molecule.

Acidification of the emulsion results in progressive precipitation of phosphatidyl serine as free acid. This precipitation is complete at about pH 1.5 (0.05 N HCl).

The insolubility of the free acid in water is made use of to prepare base-free phosphatidyl serine. The method is as follows.

3 gm. of phosphatidyl serine are emulsified with 150 cc. of water and to the emulsion are added 15 cc. of N HCl. The precipitate that forms is centrifuged off and washed once with 0.1 N HCl. The washed precipitate is transferred to a cellophane sausage casing and dialyzed against distilled water in the ice box for 3 days, the outside liquid being renewed six times. In the course of dialysis some of the precipitate goes back into emulsion. From the contents of the dialysis sack base-free phosphatidyl serine is obtained either by lyophilization or by precipitation with a large excess of alcohol (four to six times as much alcohol as water). In either case the dry substance is taken up in 10 cc. of CHCl_3 and 70 cc. of acetone are added

TABLE II
Analysis of Base-Free Phosphatidyl Serine

Component	Preparation Oct. III	Preparation Nov. III	Preparation Sept. IIIBII	Preparation Jan. III	Calculated for $\text{C}_{42}\text{H}_{80}\text{O}_{10}\text{NP}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C	63.34	63.89	63.53	63.5	63.9
H	9.92	9.72	9.87	9.82	10.1
P	3.89	3.83	3.96	3.86	3.92
N	1.74	1.80	1.81	1.69	1.78
Base	1.0	0.5	0.1	0.4	0.0
COOH-N	1.59	1.65	1.68	1.59	1.78

to it. The precipitate that forms is collected and dried, 2.3 gm. of a light tan loose powder being obtained. After being dried to constant weight over CaCl_2 in a vacuum, it still contains about 1.80 per cent water. For instance, 255.54 mg. of base-free phosphatidyl serine that had reached constant weight *in vacuo* over CaCl_2 showed a loss of weight of 4.64 mg. (*i.e.*, 1.80 per cent) on being heated in a vacuum at 80° to constant weight. The dried product, when placed in a vacuum desiccator over CaCl_2 , regained its former weight in 24 hours.

The results of chemical analyses of several preparations are given in Table II. They agree closely with values calculated for oyleylstearyl-glycerolphosphoryl serine ($\text{C}_{42}\text{H}_{80}\text{O}_{10}\text{NP}$).

After drying, base-free phosphatidyl serine forms emulsions in water with great difficulty. It is only on addition of the amount of bases (KOH, NaOH) required to reform the K or Na salt (1 equivalent of base per atom of P) that an aqueous emulsion is easily obtained.

Effect of Storage on Phosphatidyl Serine

On storage *in vacuo* in the dark at room temperature phosphatidyl serine does not change its elementary composition, but some sort of rearrangement takes place in the molecule, so that the concentration of carboxyl N drops sharply. $\text{NH}_2\text{-N}$ decreases to a much lesser degree. The results of analyses on one preparation (Nov. III) stored under the conditions given above were as follows: COOH-N : original 1.62 per cent; after 3 months 1.39 per cent; after 5 months 1.32 per cent; after 10 months 1.25 per cent; after 19 months 1.20 per cent; after 26 months 1.14 per cent. On the other hand, $\text{NH}_2\text{-N}$, which was 1.70 per cent originally, was 1.37 per cent after 26 months. These changes with storage are much less marked in the case of base-free preparations. For instance, base-free Preparation Nov. III, containing originally 1.65 per cent COOH-N and 1.80 per cent $\text{NH}_2\text{-N}$ was found to contain 1.50 per cent COOH-N and 1.70 per cent $\text{NH}_2\text{-N}$ after 26 months storage. The fact that the $\text{NH}_2\text{-N}$ values decrease to a much lesser degree than carboxyl N values suggests that the $-\text{COOH}$ group is mainly involved in the observed change.

Storage in a CHCl_3 solution at -72° (dry ice box) appears to be more satisfactory. Preparations stored this way for 18 months have shown no change in composition.

Isolation of L-Serine from Phosphatidyl Serine

2.25 gm. of phosphatidyl serine containing 1.42 per cent carboxyl N were freed of base by emulsification in water and precipitation of the phosphatide by addition of HCl up to 0.1 N concentration. The precipitate was washed once with 0.1 N HCl and next hydrolyzed by boiling 6 N HCl under reflux for 3 hours. After cooling, the hydrolysate was freed of fatty acids by filtration, and the filtrate was concentrated to 4 cc. volume. To it were added 800 mg. of *p*-hydroxyazobenzene-*p*-sulfonic acid, which was dissolved by heating in the boiling water bath. Crystals formed on standing overnight in the ice box were centrifuged in the cold and washed twice with 3 cc. of ice-cold water. The washed crystals were recrystallized twice from 4 cc. of water.

After drying at 100° the crystals weighed 710 mg. On analysis they proved to be serine *p*-hydroxyazobenzene-*p*-sulfonate. They accounted for 80.7 per cent of the carboxyl N present in the starting material. The results were as follows:

$\text{C}_{15}\text{H}_{17}\text{O}_7\text{N}_2\text{S}$.	Calculated.	C 47.0,	H 4.44,	carboxyl N 3.66,	base 0.00
	Found.	" 46.7,	" 4.39,	" " 3.62,	" 0.3

569 mg. of this compound were dissolved in water, lead acetate was added to the solution, the lead salt removed by filtration, and the filtrate freed of traces of *p*-hydroxyazobenzene-*p*-sulfonic acid with charcoal.

The filtrate from the charcoal was dried, the residue dissolved in 2 cc. of water, and 20 cc. of alcohol were added to the solution. The crystals formed on standing overnight in the ice box were collected and dissolved in 0.5 cc. of water, and 10 cc. of alcohol were added to the solution. After standing overnight in the ice box, the crystals that formed were collected, dried, and analyzed.

The crystals weighed 142 mg. On analysis they proved to be L-serine, and accounted for 91 per cent of the serine present in the serine *p*-hydroxy-azobenzene-*p*-sulfonate or 73.6 per cent of the carboxyl N present in the starting phosphatidyl serine. The results were as follows:

$C_2H_7O_2N$. Calculated.	C 34.2, H 6.67, N 13.32, carboxyl N 13.32
Found (corrected for 0.95% base).	" 34.15, " 6.55, " 13.22, " " 13.22

Rotation—A solution in 1 N HCl containing 47 mg. of crystals per cc showed in a 1 dm. tube a rotation of $+0.67^\circ$ with sodium light; $\alpha_D^{20} = 14.3^\circ$. Fischer and Jacobs give $+14.5^\circ$ (22).

Isolation of Glycerophosphoric Acid from Phosphatidyl Serine

3.35 gm. of phosphatidyl serine (Preparation May III-41) were emulsified in 150 cc. of H_2O , and 15 cc. of N HCl were added to the emulsion. The resulting precipitate was centrifuged, washed once with 0.1 N HCl, and then hydrolyzed with 6 N HCl for 100 minutes in the boiling water bath. After cooling, the hydrolysate was filtered and the filtrate evaporated to dryness in a vacuum. The dry residue was taken up in 50 cc. of water and the solution treated with 1 gm. of Ag_2O after the addition of 0.5 cc. of concentrated acetic acid. The precipitate was filtered off and the silver was removed with hydrogen sulfide. The silver sulfide was filtered off, after which the filtrate was evaporated to dryness. The residue was dissolved in 30 cc. of H_2O and brought to pH 10 by the addition of 12 cc. of saturated $Ba(OH)_2$. The precipitate that separated was centrifuged off and washed twice with water. To the clear solution an equal volume of alcohol was added and the precipitate that separated was centrifuged off and washed once with 50 per cent alcohol and dried in a vacuum at 142° to constant weight. It weighed 831.2 mg. On analysis it was found to contain 11.1 per cent P, 32.5 per cent glycerol, 32.76 per cent Ba, and 0.2 per cent N. It accounted for 81 per cent of the P present in the starting material. It appeared to be barium glycerophosphate mixed with acid barium glycerophosphate.

Four precipitations from water solution, by addition of an equal amount of alcohol, brought the nitrogen concentration down to 0.03 per cent. Finally, the Ba salt was dissolved in 50 cc. of H_2O , the solution was made

strongly alkaline by addition of 10 cc. of saturated aqueous $\text{Ba}(\text{OH})_2$ solution, and an equal volume of alcohol added to it. The alcohol-insoluble precipitate was centrifuged, washed twice with cold 50 per cent alcohol, dried, redissolved in 30 cc. of water, and CO_2 bubbled through the solution. A slight precipitate was removed by centrifugation and to the supernatant was added an equal volume of methyl alcohol. The precipitate that formed was centrifuged, washed with 50 per cent methyl alcohol, and dried to constant weight at 142° .

575 mg. of material were thus obtained. On analysis it proved to be Ba glycerophosphate. The analytical results were as follows:

$\text{C}_2\text{H}_5\text{O}_4\text{P Ba}$.	Calculated.	C 11.73,	P 10.1,	Ba 44.5,	glycerol 29.3
	Found.	" 11.69,	" 10.05,	" 44.6,	" 28.7

Estimation of Fatty Acids

Phosphatidyl serine was saponified with 8 per cent alcoholic NaOH by refluxing for 4 hours. After cooling, the solution was nearly neutralized with hydrochloric acid and concentrated to dryness *in vacuo*. The residue was acidified with N HCl and the fatty acids were extracted with four successive portions of ether. The combined ether extracts were washed three times with equal volumes of water, and the washed ethereal extract was evaporated to dryness. By this method, saponification appeared to be complete in 4 hours, the same results being obtained for periods of saponification of 4 and 8 hours.

A number of preparations were thus analyzed. The purest ones (containing 97 per cent of total N as COOH-N) yielded 68.6 per cent of the weight of the starting material as fatty acids, neutral equivalent 283; (moles of fatty acid)/(atoms of P) = 2.00. The theory for the K salt of phosphatidyl serine assumed to contain oleic and stearic acid radicals is 68.8 per cent of the starting material as fatty acids and the neutral equivalent for an equimolar mixture of oleic and stearic acids is 283. Preparations of lesser purity yielded fatty acids of higher neutral equivalent (up to 291).

Isolation of Component Fatty Acids—4 gm. of phosphatidyl serine were emulsified in 300 cc. of H_2O , and 25 cc. of 19.2 N NaOH were added to the emulsion. The solution was immersed in a boiling water bath for 6 hours. After cooling, the solution was acidified with HCl to pH 1.5 and allowed to stand overnight in the ice box. The next morning it was extracted four times in succession with an equal volume of ether each time. The ethereal extracts were combined and washed twice with an equal volume of water, after which the solution was evaporated to dryness. The residue weighed 2.7 gm.

The fatty acids were separated into saturated and unsaturated acids by the method of Twitchell (23) as follows: The residue was dissolved in 80 cc.

of alcohol in a 100 cc. centrifuge tube immersed in a water bath at 75°, and to the hot solution were added in succession 0.5 cc. of acetic acid and 7 cc. of a 25 per cent aqueous neutral lead acetate solution. A small amount of precipitate settled to the bottom. The clear supernatant solution was transferred to another centrifuge tube in the same bath and then the bath was allowed to cool. Next morning the clear supernatant was decanted and the precipitate was washed with alcohol. The combined washing and supernatant solutions were evaporated to dryness. The residue was dissolved in 30 cc. of ether and the ethereal solution allowed to stand overnight in the ice box. The small amount of precipitate formed was filtered off and discarded. The filtrate was evaporated to dryness and the residue dissolved in 40 cc. of methyl alcohol, after which the lead was removed with hydrogen sulfide. The filtrate from the lead sulfide was evaporated to dryness. The residue was an oil which contained some white crystals. It weighed 1250 mg.

The oily residue was treated with 30 cc. of petroleum ether. Most of it went into solution. After standing overnight in the ice box, the petroleum ether solution was filtered and the filtrate evaporated to dryness. The residue was a clear oily liquid and weighed 1100 mg. The neutral equivalent was 283 and the iodine number 87.0. It appeared to be slightly impure oleic acid. Attempts to isolate analytically pure oleic acid from it have failed. The amount of impure oleic acid obtained amounted to 79.5 per cent of the amount present in the starting material, according to the postulated formula.

The alcohol-insoluble lead soaps were reprecipitated from 60 cc. of hot alcohol (to which 0.5 cc. of acetic acid had been added), the solution being allowed to cool over a period of 2 hours. After standing overnight at room temperature, the precipitate formed was collected by centrifugation and washed with 95 per cent alcohol.

After drying in a vacuum, the lead soaps were transferred to a separatory funnel with ether and the ethereal suspension washed with dilute nitric acid and then four times with water, after which the ethereal solution was evaporated to dryness. The residue was a crystalline mass which weighed 1100 mg., m.p. 65°. Neutral equivalent 279; iodine value <1.0. It was assumed that the material was impure stearic acid. The residue was dissolved in 80 cc. of hot alcohol. On cooling, a small amount of precipitate separated which was filtered off and the filtrate evaporated to dryness, after which the residue was dissolved in 40 cc. of hot alcohol. On cooling, a small precipitate formed. The solution was filtered, evaporated to dryness, and the residue dissolved in hot alcohol. No precipitate formed on cooling. The solution was evaporated to dryness.

The crystalline residue weighed 950 mg. On analysis it proved to be pure stearic acid. The results were as follows:

$C_{18}H_{34}O_2$.	Calculated.	C 76.0,	H 12.72,	Base
	Found.	" 75.8,	" 12.65,	" 0.1

M.p. 69.3°; mixed m.p. with stearic acid, 69.3°; neutral equivalent 284.0.

The amount of pure stearic acid obtained represents 69 per cent of the amount present in the starting material, according to the postulated formula.

SUMMARY

1. A method is described for the isolation of phosphatidyl serine of at least 92 per cent purity.

2. As cleavage products, glycerophosphoric acid, L-serine, and fatty acids have been isolated in molecular proportions of 1:1:2. Fatty acids present appear to be mainly stearic acid and oleic acid.

3. Phosphatidyl serine reacts with HNO_3 and ninhydrin as an α -amino acid, which shows that both the $-COOH$ and the $-NH_2$ groups are free. It does not react with HIO_4 , which shows that either its $-NH_2$ or its $-OH$ group is combined. Therefore, it appears that the combination of serine in the phosphatidyl serine molecule is through its $-OH$ group.

4. It is concluded that the structure of phosphatidyl serine is that of stearyl-oleylglycerylphosphoryl serine. The results of analyses are found to agree with the values calculated from the postulated formula.

5. As isolated by neutral solvents, phosphatidyl serine is obtained as a K and Na (the former being the most abundant) salt, (equivalence of base)/-(atoms of P) = 1.00. The inorganic cations can be removed by treatment with 0.1 N HCl.

6. Phosphatidyl serine studied in this paper represents 30 per cent of total lipid carboxyl N in brain. Other preparations of phosphatidyl serine can be obtained in which Na is the most abundant base.

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DURATION OF RENAL ISCHEMIA REQUIRED TO PRODUCE UREMIA*

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Bywaters and his colleagues (1) early in the late war drew attention to the fact that shock from crush injuries and other traumata is not infrequently followed by renal failure that persists after other signs of shock have disappeared and can result in fatal uremia. Experiments from this laboratory, presented in a preliminary report in 1944 (2), and later detailed (3), showed that severe hemorrhagic or traumatic shock in dogs could cause almost complete cessation of renal blood flow. Lauson, Bradley and Cournand (4) at about the same time reported decreased renal blood flow in human cases of shock. Since these effects could occur while the arterial blood pressure was as high as 80 to 100 mm. it appeared that the decrease in renal blood flow was due to vascular constriction (2, 3). Of the different hypotheses that had been advanced concerning the cause of the renal damage produced by shock (see Lucké, 5), the demonstration of decreased renal blood flow supported the hypothesis that renal ischemia existing during the shock state is a cause of subsequently persisting renal failure (2, 3).

To test this hypothesis further, renal ischemia was produced in dogs (2) by ligating the renal arteries for varying periods, and the after effects on renal function were observed. Ischemia of two-hours' duration was followed by temporary depression of urea clearance to nearly zero, but gradual recovery followed during a period of two or three weeks, and renal function might be completely restored. Ischemia of over four-hours' duration uniformly caused irreversible renal injury leading to death in uremia in four to eight days. Histological examinations by Dr. Jean Oliver showed that the glomeruli appeared to be normal, but that, in the distal tubules, there were changes resembling those seen in cases of uremia following the crush syndrome.

The present paper presents details of, and additions to, the original experiments performed to determine the duration of renal ischemia necessary to cause irreversible damage. As will be seen, dogs survived renal ischemia for two hours.

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Depending on environmental temperature, nutritional state of the animal, etc., three hours of renal ischemia might or might not be fatal.

At the time these experiments were undertaken, except for one paper by McEnery, Meyer and Ivy (6), no data could be found in the literature to indicate the duration of renal ischemia required to cause irreversible injury of the kidneys in dogs. McEnery *et al.* state, "Dogs may or may not survive a 30- to 60-minute period of ischemia of both kidneys". Scarff and Keele (7), confirmed recently by Badenoch and Darmady (8) found that one or two hours of occlusion of the renal artery produced irreversible renal damage in rabbits. To judge by comparison with our results, irreversible damage to the kidneys is caused by ischemia of somewhat shorter duration in rabbits than in dogs.

METHODS

1. *Method of producing renal ischemia.* The following procedure was applied with aseptic precautions to a series of female dogs under sodium pentobarbital anesthesia.

Through a midline abdominal incision the right kidney was excised. The left kidney was mobilized by dividing between silk ligatures the parietal peritoneum and the accompanying capsular vessels about one inch from the kidney. The renal artery was dissected free down to its origin from the aorta for a distance of $1\frac{1}{2}$ to 2 cm. Two serrefines, with jaws covered by rubber tubing and with springs of sufficient strength to obliterate the arterial lumen without damaging the intima, were then applied to the renal artery. The time of application of the serrefines was noted and the wound closed in layers with silk. In none of our experiments was the renal vein or the ureter obstructed. The presence of the ureteral vein and artery was noted at operation but it was felt unwise to ligate the ureteral artery even though in some instances it was obvious that some blood was still entering the kidney through this small vessel.

Following the removal of the serrefines the kidney was tacked in its original position by mattress sutures placed through the renal peritoneum and the dorsal abdominal wall at the four poles of the kidney. The abdominal incision was closed with layered silk sutures.

2. *Chemical determinations.* The blood urea nitrogen concentration was followed in all experiments by the manometric hypobromite method of Van Slyke and Kugel (9). In experiments performed in the months of June and July 1944 continuous 24-hour urea clearances were also performed. Urine urea determinations were made by the method of Van Slyke and Cullen (10). In this series of experiments urea clearances were performed before the serrefines were applied to the renal artery and for the two hours after release of the serrefines, at which time the animal was still under sodium pentobarbital anesthesia. Subsequently the animals were placed in metabolic cages and 24-hour collections of urine obtained. The urea clearances were followed until the death of the animal or until the clearance had returned to the normal range.

RESULTS

Experiments Performed in January, February and April 1943

1. *Renal ischemia for one hour.* Six dogs subjected to renal ischemia for one hour survived more than 30 days.

2. *Renal ischemia for two and three hours.* Six dogs were subjected to renal ischemia for two hours and six dogs were subjected to renal ischemia for three hours. One animal in this series died on the fourth post-operative day with a large *B Coli* abdominal abscess, the remainder of the dogs in this series survived more than 30 days.

3. *Renal ischemia for four hours.* Of six dogs subjected to renal ischemia for four hours, three lived for more than 30 days; three dogs died after 4, 4 and 10 days with blood urea nitrogen concentrations at death of 157, 182 and 320 mgm. per 100 cc., respectively.

4. *Renal ischemia for six hours.* Two dogs were subjected to renal ischemia for six hours. Both died in uremia after 4 and 10 days, respectively.

5. *Bilateral nephrectomy.* Two dogs were subjected to removal of both kidneys. They died on the fourth and eighth day, respectively, in uremia, verifying the observations of Hoff, Smith and Winkler (11).

Experiments Performed in June and July 1944

1. *Renal ischemia for two hours.* Five dogs were subjected to renal ischemia for two hours; all survived for over 30 days.

2. *Renal ischemia for three hours.* Of five dogs subjected to renal ischemia for three hours, two dogs survived for over 30 days; the three dogs that died survived 6, 9 and 15 days and the blood urea nitrogen concentrations at death were 374, 361 and 462 mgm. per 100 cc., respectively.

In our first series of experiments with renal ischemia of three hours' duration it will be noted that the experiments were performed in the winter and spring of the year when the environmental temperature was never above 80° F., while the second series of experiments were performed in the summer months when the environmental temperature was often above 95° F. and the humidity was also greatly increased. It appears that the increased environmental temperature and humidity in the summer months may have been responsible for the increased mortality of the dogs subjected to three hours of renal ischemia during this time of the year (cf. Lusk, 12, p. 151-155).

Blood Urea Changes

In figure 1 the plasma urea nitrogen concentration changes in one animal which had a bilateral nephrectomy are compared with the plasma urea nitrogen changes in three dogs subjected to renal ischemia of three hours' duration. The nephrectomized dog, K-7, died on the fifth day. K-91 and K-90, subjected to three hours of renal ischemia, died on the sixth and fourteenth day, respectively. These experiments were performed in the summer months. K-11, subjected to

three hours of renal ischemia in April, survived for over 30 days. The urea concentration curve of *K-11* is quite typical of the type of curve seen in all animals that survive. It will be noted in this animal that the blood urea rose at a steady rate for three days then remained practically stationary for two days and subsequently decreased. Histological studies of the kidneys of dogs subjected to renal ischemia for one, two and three hours will be reported elsewhere by Dr. Jean Oliver. However, we can state that the histological evidence for regeneration of

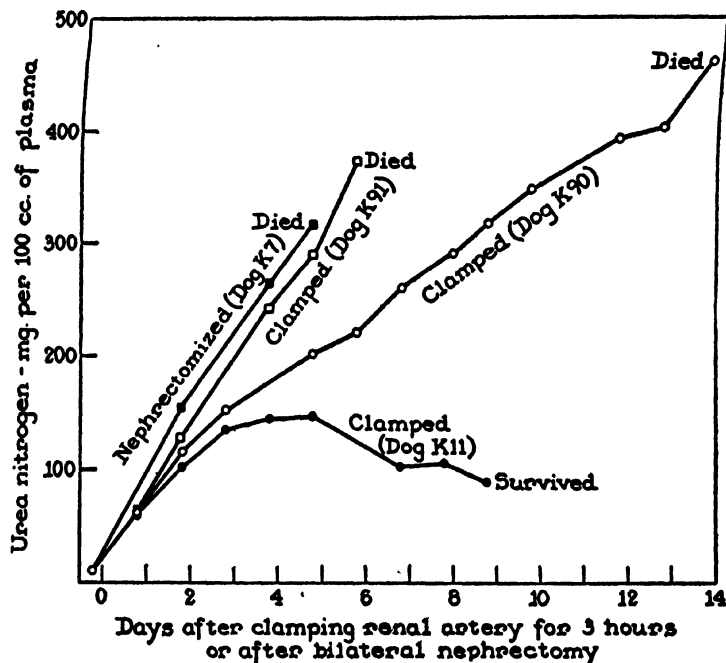


FIG. 1. COURSE OF CHANGES IN PLASMA UREA NITROGEN CONCENTRATION in a bilaterally nephrectomized Dog (*K 7*) compared with changes in three dogs subjected to renal ischemia of three hours' duration.

kidney tissue subjected to renal ischemia does not appear before the third day after induction of ischemia.

Urea Clearance Studies Following Renal Ischemia

Figure 2 compares the urea clearance and plasma urea nitrogen concentration in *dog K-89* subjected to two hours of renal ischemia on June 28, 1944. There is a steady improvement of the urea clearance in the first three days following induction of ischemia, although during this time there is a steady increase in the blood urea nitrogen. Although renal function was improving from the day of operation, three days were required before the improvement progressed far enough to stop the rise of blood urea. The urea clearance rose in about 35 days

of Rhoads, Alving, Hiller and Van Slyke (13) to be normal for a one-kidney dog of its size.

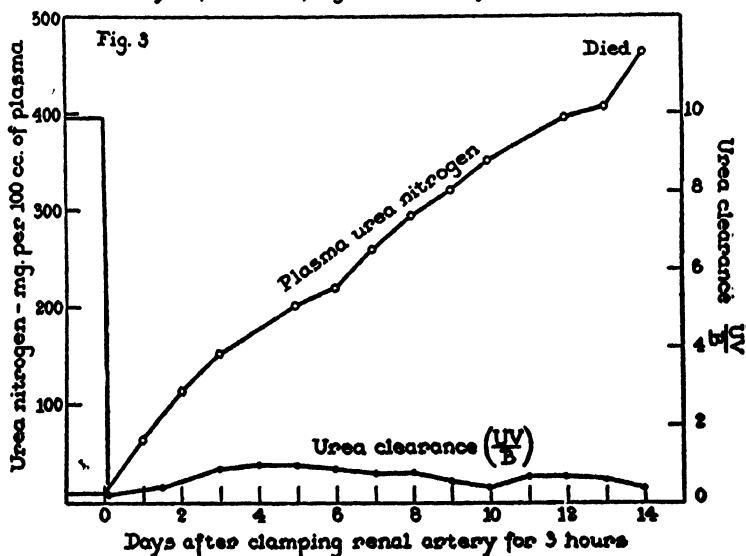
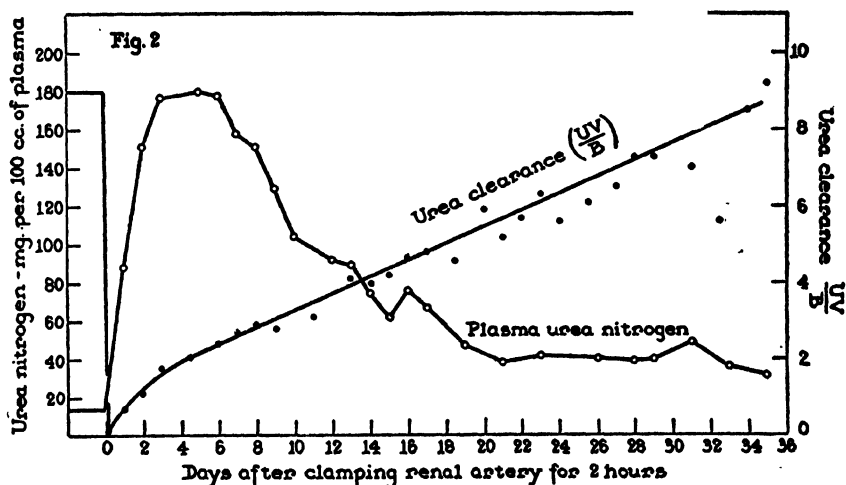


FIG. 2. COURSE OF CHANGES IN UREA CLEARANCE and plasma urea nitrogen concentration in Dog K 89 after two hours of renal ischemia.

FIG. 3. COURSE OF CHANGES IN UREA CLEARANCE and plasma urea nitrogen concentration in Dog K 90 after three hours of renal ischemia.

Figure 3, K-90, compares urea clearance with plasma urea nitrogen concentration in a dog which survived for 14 days after being subjected to three hours of renal ischemia. In contrast to K-89, this animal after the first three days following

three hours of renal ischemia showed no tendency for improvement of the urea clearance, after the fifth day there was a slow downward trend.

SUMMARY

1. Dogs with the right kidney removed uniformly survived clamping of the left renal artery for two hours. Some dogs survived clamping of the renal artery for three or four hours. Death in uremia regularly ensued with longer clamping of the renal artery.

2. In dogs subjected to three hours of renal ischemia the mortality was greater during summer, with high environmental temperature and humidity, than during winter, with low temperature and humidity.

3. After the clamp was removed from the renal artery, the urea clearance was extremely low, of the order of one to 10 per cent of normal, and blood urea began to rise rapidly. If recovery ensued, a steady rise of renal function occurred as measured by the urea clearance, and the clearance eventually reached a normal level, although a month was sometimes required for it to do so. If recovery did not take place, renal function remained at a low level, with progressive rise in blood urea nitrogen terminating in uremia and death.

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EFFECT OF 20, 60 AND 120 MINUTES OF RENAL ISCHEMIA ON GLOMERULAR AND TUBULAR FUNCTION*

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In other papers (1, 2) it has been demonstrated that renal ischemia of two-hour duration, caused by clamping the renal arteries of dogs, although not quite sufficient to cause irreversible injury, was followed by a period during which the urea clearance was depressed to a small fraction of its normal value, the blood urea nitrogen rose, and normal function was regained only in the course of two or three weeks.

The fall in urea clearance might be due to 1) decrease in the renal blood flow, or 2) decrease in the proportion of plasma water, with its urea and other crystalloid solutes, filtered in the glomeruli or 3) increase in reabsorption of urea in the tubules, such as might occur if the tubular walls were so devitalized by the ischemia that they could not prevent back diffusion of urea with reabsorbed water.

In an attempt to determine which of the three factors were responsible, experiments of the following type were performed. The right kidneys of female dogs were removed, and the dogs were infused with a solution of para-amino hippuric acid (hereafter designated as PAH) and creatinine, as described in a previous paper (3). The left kidneys were subjected to ischemia by clamping the renal arteries for periods of 20, 60 or 120 minutes. At intervals up to two hours after removal of the clamps blood was drawn simultaneously from the renal artery and the renal vein, analyses of the plasma from these bloods were made for creatinine and PAH and the rates of excretion of both substances in the urine were determined.

Of the three factors considered as possible causes for the persisting low urea clearance after renal ischemia, the results of the experiments could be expected to give indications as follows.

1. Renal blood flow could be calculated from the PAH values by the Fick principle, as $(\text{PAH excreted per minute})/(\text{PAH removed by the kidneys from one cc. of renal blood})$. This principle of measuring renal blood flow was introduced by Van Slyke, Rhoads, Hiller and Alving (4), who used urea as the measured

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research. The results were in part presented in 1944 by the authors and collaborators (1) in a report on the effects of shock on the kidney. The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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excretory substance. However, since only about 12 per cent of the blood urea is extracted by the kidneys, while an average of 87 per cent of plasma PAH is extracted (1, 3, 5) the PAH extraction can be measured more accurately. Previous experiments (1, 3) have shown that consistent values for renal plasma flow are obtained by the Fick principle, with PAH as excretory substance.

2. The fraction of plasma water filtered could be estimated from the fraction of creatinine extracted from the renal plasma, *provided* that the ischemia does not so injure the tubules that they permit some of the creatinine filtered in the glomerulus to diffuse back into the blood with the water reabsorbed from the tubular umina. Previous studies (6) have indicated that creatinine in the normal dog is excreted entirely by glomerular filtration, without significant tubular reabsorption.

3. The functional state of the tubular cells could be indicated by the completeness with which the kidneys remove PAH from the renal blood plasma. Since normally an average of 87 per cent is removed (1, 3) while only 20 to 30 per cent can be considered to be filtered in the glomerulus (6), it follows that the greater part of the PAH removed from the plasma is normally excreted by the tubules. If, after renal ischemia, the tubules can at once resume extraction of PAH so complete that 87 per cent is removed from the renal plasma, it can be assumed as probable that the tubular cells are undamaged. If, however, there is a severe decrease in the PAH extraction, it may be taken as evidence of deranged tubular function and would suggest the possibility that the renal malfunction may include increased permeability to back diffusion of urea from the tubular lumina to the blood. Normally the tubules limit back diffusion of urea to about 40 per cent of that filtered (6, 7).

METHODS

1. *Operative and experimental procedures.* Female dogs were used. After anesthetizing with sodium pentobarbital, the spleen and right kidney were excised through a midline abdominal incision. The peritoneum and adventitia overlying the left renal vein were then dissected free from the vein so that samples of blood could be readily obtained. The parietal reflection of renal peritoneum was then excised between the upper and lower poles of the left kidney to permit exposure and dissection of the artery for a distance of one half to two cm., sufficient to accommodate the serrefines applied subsequently for the production of renal ischemia. A urethral catheter was inserted into the bladder and strapped in place. A ureteral catheter was inserted into the fore-limb vein and was connected to a continuous intravenous infusion pump, for the infusion of the solution of creatinine and PAH (3).

The technique for obtaining blood and urine samples was as described by Phillips *et al.* (3). Blood was drawn from the renal vein with a syringe provided with a curved No. 20 hypodermic needle; the needle was bent in a 90° curve so that its point was at right angles to the syringe axis. This facilitated puncturing the renal vein to obtain the sample. Care was taken to avoid undue tension on the kidney, its vessels and the ureter at the time of sampling, and in addition

care was taken to avoid any tension on the abdominal vena cava at the time of sampling.

In these experiments it was of interest to follow the clearance and extraction of creatinine and PAH immediately after release of the serrefines applied to the renal artery. Consequently the peritoneum and capsular vessels at the upper and lower pole of the kidney were left undivided, to insure that the kidney would remain in its original position and that kinking of the ureter, renal vein and renal artery would be minimized. As pointed out in the preceding paper (2), some blood does enter the kidney by way of the capsular vessels (not divided in these experiments) and in some animals by way of the ureteral artery. However, the amount of such vascular supply is small compared to the supply obtained through the renal artery.

Priming, followed by sustaining, infusions of creatinine and PAH were given, and one hour was allowed for attainment of equilibrium of creatinine and PAH between plasma and cells (3). One hour after start of the sustaining infusion three urine samples, collected during successive periods of 10 to 15 minutes each, were obtained. Blood was drawn from the femoral artery before the start of the first urine period and at the end of the third urine period. A sample of renal venous blood was obtained in the middle of the second urine period. After these control observations serrefines with rubber-covered jaws were applied to the renal artery for varying periods of time, which, in 3 series of experiments were approximately 20 minutes, 60 minutes and 120 minutes respectively. Ten minutes after release of the serrefine a sample of arterial blood was drawn and urine samples were obtained every 15 minutes for the subsequent two or more hours. Arterial blood samples were obtained at frequent intervals from the femoral artery. Renal venous blood samples were obtained at approximately 20, 60 and 120 minutes after release of the serrefines.

During application of the serrefines the sustaining infusion was discontinued. When renal ischemia was maintained for 120 minutes bladder washings for 110 minutes of this time were obtained. The smallness of the amounts of PAH found in these bladder washings, obtained during the period of ischemia, was a further check on the completeness of the renal ischemia. In two experiments both kidneys of each dog were excised and the bladder clearance of PAH (clearance estimated from PAH in bladder washings of the nephrectomized dogs) was determined with plasma PAH concentrations of 4-6 mgm. per 100 cc. The bladder clearance was found to be in the range of 0.02-0.04 cc. per minute. In the experiments here reported the PAH clearance during 120 minutes of renal ischemia were never more than 10 times the bladder clearance, indicating that the renal ischemia was nearly complete.

2. *Method of administration and analysis of creatinine and PAH.* The techniques used for administering creatinine and PAH and for analyzing plasma, blood and urine for these substances have been described previously (3). PAH concentrations found in plasma of renal venous blood were corrected as previously described (3), for diffusion of 5 per cent of the plasma PAH *in vitro* from plasma to erythrocytes during the drawing and centrifuging of the blood.

RESULTS AND DISCUSSION

Renal blood flow. The results in table 1 indicate that, after renal ischemia for periods up to two hours, when the clamps were removed from the renal arteries blood flow through the kidneys was soon reestablished. Renal plasma flows, measured 90 to 120 minutes after release of the clamps, averaged 81 to 85 per cent

TABLE 1. *Effects of clamping renal artery for varying lengths of time on subsequent renal function (one-kidney dogs)*

DURATION OF CLAMPING	DOG NO.	% OF EXCRETORY SUBSTANCE EXTRACTED FROM RENAL PLASMA						RENAL PLASMA FLOW ¹			RENAL PLASMA FLOW ESTIMATED AS 1.15 X PAH CLEARANCE		
		Para-amino hippurate			Creatinine			Before clamping	15-20 min. after release of clamp	90-120 min. after release of clamp	Before clamping	15-20 min. after release of clamp	90-120 min. after release of clamp
		Before clamping renal artery	15-20 min. after release of clamp	90-120 min. after release of clamp	Before clamping	15-20 min. after release of clamp	90-120 min. after release of clamp						
min.								cc/min.	cc/min.	cc/min.	cc/min.	cc/min.	cc/min.
20	N2	0.91	0.85	0.88	—	—	—	187	96	48	164	94	49
20	N3	0.85	0.79	0.73	0.33	0.21	0.21	48	85	85	47	97	71
20	N4	0.80	0.87	0.61	0.30	0.23	0.38	33	22	18	30	14	13
20	N9	0.90	0.90	0.72	0.17	0.22	0.26	61	58	46	63	60	38
Mean % of value before clamping		100	90	85	100	90	118	100	101	85	100	91	71
60	N6	0.76	0.39	0.50	0.33	0.15	0.20	67	37	66	59	17	38
60	N7	0.83	0.67	0.71	0.22	0.16	0.25	99	98	76	94	29	46
60	N8	0.81	0.31	0.38	0.22	0.05	0.15	96	67	72	90	24	31
Mean % of value before clamping		100	57	66	100	47	72	100	75	84	100	29	49
120	K69	0.90	0.11	0.11	0.23	0.02	0.02	87	57	76	90	7	9
120	K67	0.91	—	0.20	0.19	—	0.05	93	—	56	96	—	13
120	K68	0.94	—	0.43	0.22	—	0.14	42	—	36	46	—	17
120	K70	0.92	0.16	0.13	—	—	—	116	52	90	122	9	14
Mean % of value before clamping		100	—	24	100	—	37	100	—	81	100	—	18

¹ Renal plasma flow measured as (mgm. PAH excreted per min.) / (mgm. PAH extracted from 1 cc. of plasma).

of the pre-ischemic flows, whether the period of ischemia had been 20, 60 or 120 minutes. The decrease of urea clearance nearly to zero, noted previously (1) as a sequence of clamping the renal artery for two hours, was evidently not due to failure of blood flow to return to the kidneys after the clamps were removed.

PAH extraction and tubular damage. The PAH extraction values indicate that tubular function was severely deranged by two hours of ischemia. The damage was progressive with the duration of the ischemia, as indicated by the progressive

decrease in the fraction of PAH extracted from the renal plasma; this fraction averaged 85 per cent of its pre-ischemic value after 20 minutes ischemia, 66 per cent after 60 minutes and 24 per cent after 120 minutes of ischemia, the extractions being averages measured 90–120 minutes after release of the arterial clamps. This functional evidence of tubular damage agrees with the results of histological examination of the kidneys by Dr. Jean Oliver, mentioned in a previous report (1).

Tubular reabsorption as a cause of renal failure after renal ischemia and shock. The functional effects of tubular injury have been studied by Richards (8), who observed *in vivo* the activities of the nephrons of frogs that had been poisoned by mercury and other nephrotoxic substances. He found that in the glomeruli blood flow and filtration went on at a fully normal rate, but that the filtrate was completely absorbed from the tubular lumina, so that no urine reached the bladder. Anuria resulted, not from failure of filtration, but from complete reabsorption of the filtrate. The poisoned tubular walls permitted unselective diffusion of the entire filtrate, presumably drawn by the osmotic attraction of the plasma proteins, back into the circulation. The tubular walls apparently had become devitalized, so that they acted like inert permeable membranes.

In view of the absence of evidence of marked glomerular or vascular injury and of the positive evidence, functional and histological, of tubular injury, it appears probable that tubular reabsorption of the type observed by Richards (8) causes the urea retention that follows sufficiently prolonged periods of renal ischemia, as exemplified by figure 1 (reversible) and figure 2 (irreversible) of the preceding paper (2).

Lucké (9) has noted in kidneys from cases of uremia following shock from war injuries that the type of tubular injury was identical with that observed in cases injured by nephrotoxic poisons, and points out, from analogy with Richards' observations, that post-shock uremia is probably the effect of tubular injury caused by ischemia suffered during the shock, the renal failure being due to unselective tubular reabsorption of the type observed by Richards. Badenoch and Darmady (10) have compared side by side sections from the kidneys of patients dying in post-shock uremia with sections from the kidneys of rabbits in which uremia was caused by two-hour ligation of the renal arteries, and have found the type of tubular lesions practically identical. Results in previous papers from this laboratory (3) have shown that hemorrhagic and traumatic shock of severe grade causes nearly complete cessation of blood flow through the kidneys of dogs.

These observations, taken with the present data showing that ischemia prolonged over periods of hours comparable to the duration of shock in severe untreated cases produces progressive damage to tubular function, indicate the probability that post-shock uremia is caused by reabsorption of excretory products through damaged tubular walls. It also appears, from Lucké's (9) observation of the identical nature of the tubular lesions in post-shock uremia and in poisoning by sulfonamides and other nephrotoxic agents, that ischemia is one of many nephrotoxic agents that produce a common picture of tubular damage with resultant renal failure from tubular reabsorption.

Cause of the decreased extraction of creatinine from the renal plasma after ischemia.

In the kidneys of the normal dog excreting ordinarily large volumes of urine there is evidence that creatinine and inulin do not pass to a measurable extent in either direction through the tubular walls, so that the fraction of either removed from the renal plasma is the fraction that is filtered out in the glomeruli (6, 11). It has been rather generally assumed that these relations hold also in abnormal kidneys, the clearance of creatinine or inulin in dogs and inulin in man serving as a measure of glomerular filtration. On such an assumption, a great decrease in the extracted fraction of creatinine, such as is seen in table 1 after two-hour ischemia, without a commensurate decrease in blood pressure, would be taken as evidence of decreased filtration due to decreased blood pressure in the glomerular capillaries, the fall in pressure being attributable to dilatation of the efferent vessels from the glomeruli.

However, in view of the evidence of tubular damage in these animals, of Richards' (8) evidence that such damage can cause abnormal tubular reabsorption, and in view of the lack of evidence of efferent dilatation, it appears more probable that the decrease in the filtered fraction of creatinine is attributable to back diffusion of creatinine through the injured tubular walls into the circulation. The condition appears to be similar to that found by Richards, Westfall, and Bott (12) in a dog with kidneys previously damaged by uranium. Creatinine clearances were consistently lower than inulin clearances. The results were interpreted by Richards *et al.* as evidence of back diffusion of creatinine through the injured tubular walls, inulin back diffusion being less because of lesser diffusibility of the large inulin molecule.

Interpretation of clearances as measures of renal blood flow and glomerular filtrate in conditions of tubular injury. *Renal blood flow.* Smith and his colleagues have shown for normal men and dogs that the clearances of diodrast (13) and PAH (14) approach so nearly to otherwise estimated values of the renal plasma flow that it is evident that their extractions from the renal plasma must be nearly complete, and that their clearances, in terms of cc. of plasma cleared of diodrast or PAH per minute, may therefore be assumed to approximate the volume of plasma flowing through the kidneys. These conclusions have been confirmed for subjects with normal kidneys; in both dogs (1, 3) and men (5) simultaneous determinations of PAH in arterial and renal venous plasma have shown that an average of 87-88 per cent of the PAH is normally extracted by the kidneys. Hence, when the kidneys are normal, one can estimate the renal plasma flow as 1.15 times the plasma PAH clearance, usually with but a small per cent error (3). This relation was shown to hold in acute hemorrhagic and traumatic shock so severe that the renal blood flow was temporarily reduced to a small fraction of normal (3). However, the results in the present paper show that when renal ischemia is sufficiently complete and prolonged to cause severe tubular injury the relation no longer holds. Comparison of the last three columns of table 1 with the preceding three columns shows that 1.15 times the PAH clearance approximately equaled the measured renal plasma flow in observations made before the renal artery was clamped, and after release of the clamps when the

latter had been applied for only 20 minutes. But when the clamps had been applied for two hours the subsequent values of 1.15 times PAH clearance averaged less than one fourth of the measured renal plasma flow. It is evident that when the tubules are injured PAH extraction may be so diminished that the PAH clearance indicates only a small fraction of the renal blood flow. Presumably the same limitation applies also to diodrast clearances. Smith has been careful to state that PAH and diodrast plasma clearances will fall short of renal plasma flows in proportion as the fraction of PAH or diodrast extracted from the renal plasma falls short of unity. The present results indicate the need of keeping this reservation in mind, particularly in conditions where tubular injury may be present.

Glomerular filtrate. In normal dogs there is evidence that the tubular walls neither excrete nor reabsorb creatinine and inulin (6); hence the fraction of either extracted from the renal plasma by the kidneys may be taken to be the fraction filtered in the glomeruli. On the evidence of the classical experiments of Richards and his collaborators (15) showing that plasma crystalloids and water are filtered in the same proportions that they have in the plasma, the fraction of creatinine or inulin extracted can be taken as a measure also of the fraction of plasma water filtered, as long as the tubular walls retain their normal impermeability to the creatinine and inulin. When the tubules are damaged, however, so that they cannot extract PAH with normal completeness from the plasma, it appears possible that the tubular walls may become sufficiently permeable to creatinine and inulin to permit back diffusion of these substances from the tubular lumina into the circulation, in the manner observed by Richards (8), so that the amounts excreted are much less than those filtered in the glomeruli. For reasons discussed above, we are inclined to interpret the very low extractions of creatinine noted after two hours ischemia (table 1) to tubular back diffusion. Although the lack of an independent measure of glomerular filtration makes the conclusion less certain than those reached with regard to the interpretation of PAH clearances as renal blood flows in the same experiments, it appears that interpretation of clearances of creatinine, and of such similarly excreted substances as inulin, mannitol, and thiosulfate, in terms of glomerular filtration rates is open to doubt in conditions where tubular damage may make reabsorption of these substances a possibility.¹

It is of interest in this connection that the impermeability of even normal tubular walls to creatinine, in the case of man, appears to be not absolute. Chesley (16) has found that when dehydration shrunk the urine flow of human subjects below 0.35 cc. per minute, reabsorption of all the urine solutes, including creatinine, occurred.

¹ In a publication from this laboratory (3) on the effects of acute shock on renal function of dogs, the fraction of creatinine extracted from the renal plasma was used as a measure of the filtered fraction of plasma water. In this case the assumption was presumably justified, since simultaneous determinations of the extracted fraction of PAH showed that this fraction was normal, 0.87 ± 0.04 , indicating that the degree of shock used had not yet significantly injured the tubules.

SUMMARY

Dogs have been subjected to renal ischemia by clamping the renal arteries for periods varying from 20 minutes to two hours, and the effects on the renal function have been studied by observations of the renal plasma flow and of the completeness with which para-amino hippurate and creatinine were extracted from the renal plasma during two hours after removal of the clamps.

Renal blood flow was quickly resumed at a nearly pre-ischemic rate.

The proportions of para-amino hippurate and creatinine extracted from the plasma were not markedly affected after 20-minute ischemia, but after two-hour ischemia they were reduced, the creatinine extraction, in 3 experiments, to 63, 26, and 9 per cent respectively of pre-ischemic values, the PAH extraction, in 4 experiments, to 37, 14, 11, and 10 per cent, respectively.

From the functional and histological effects of the two-hour ischemia, it appears probable that the decreases in extracted fractions were due to tubular injury, which decreased the proportion of plasma PAH excreted by the tubules, and increased tubular reabsorption of creatinine from the glomerular filtrate. The evidence supports Lucké's view that post-shock uremia is the result of tubular reabsorption of excretory products.

With tubular injury such as that caused by ischemia, PAH clearance does not serve as a measure of renal blood flow; and it appears doubtful that in the presence of such injury clearances of creatinine and similarly diffusible substances can be interpreted as measures of glomerular filtration rate.

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THE USE OF CONCENTRATED HUMAN SERUM ALBUMIN IN THE TREATMENT OF CIRRHOSIS OF THE LIVER*

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The formation of ascites in patients with cirrhosis of the liver probably depends on at least three factors: (1) hypoalbuminemia, (2) portal hypertension, and (3) increased amounts of circulating antidiuretic substance. The relative importance of these factors varies with the individual patients. Although the formation and disappearance of ascites do not correlate perfectly with levels of serum albumin in all patients, in the majority of cases the two findings are coincident and apparently related (1, 2). The use of concentrated human serum albumin in these patients for its osmotic effect would be expected to cause a diuresis. The extent to which this diuresis is produced would depend in large part upon the relative importance of the various factors of ascites production, being most marked in those patients in whom hypoalbuminemia is most specifically at fault.

The observations of Janeway (3) and Thorn (4) and their co-workers in patients with liver disease demonstrated that the albumin level of the serum could be raised to normal by repeated injections of albumin. Certain of their patients showed a loss of edema and ascites but the oncotic effects were disappointing in their experience. While the largest portion of the administered albumin did not remain in the blood stream, nitrogen balance studies showed that this protein had been retained, presumably in the tissues. The question as to whether parenterally administered albumin is utilized in building tissue protein is not

* The serum albumin used in this study was supplied in part by the Bureau of Medicine and Surgery, United States Navy, and in part by the American National Red Cross. It was prepared from blood collected by the American Red Cross from voluntary donors.

This is one of a series of investigations on serum albumin being carried out with material supplied by the American National Red Cross. As soon as sufficient data become available to justify final conclusions concerning its therapeutic value, a full report to the medical profession on the use of serum albumin in medical practice will be published.

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completely clear, however. The work of Whipple and co-workers (5, 6) in dogs has indicated that plasma protein may replace tissue protein without being broken down to its constituent amino acids. Albright and co-workers (7) by measuring phosphorus and potassium as well as nitrogen balance, have obtained evidence for the utilization of part of the injected albumin for the building of tissue protein in certain patients.

In addition to its oncotic and nutritional properties, albumin probably has other important functions in the body. It has the remarkable capacity to enter into combination with many molecules and ions in a manner different from other proteins (8). This property also has made albumin a valuable supplement to a medium for the growth of tubercle bacilli (9). The significance of the binding effect of albumin in the living organism has not been fully determined but it undoubtedly is important in the transport of various substances through the body. In view of these specific effects of albumin, it is to be expected that albumin therapy in liver disease would produce profound metabolic results.

Materials and Methods

The present study represents the results of albumin therapy in 17 patients with severe liver disease. All but two of them demonstrated ascites at the time when albumin was first administered. The diagnosis was well-established by a characteristic history, numerous physical examinations, x-ray studies of the esophagus and abdomen, and a wide variety of liver function studies. In eight of the patients the diagnosis was confirmed by the gross appearance of the liver and from sections of the liver obtained at laparotomy or autopsy. All patients were hospitalized at the Hospital of The Rockefeller Institute during the period of albumin therapy. When sufficient improvement had been accomplished by hospitalization, the patients were observed twice weekly in the Out Patient Department.

Etiology of the 15 cases of cirrhosis in this series was varied. Four gave a clear-cut history of chronic alcoholism with associated nutritional deficiencies; four were patients with cirrhosis of the liver following infectious hepatitis; in the remaining seven the etiology of the cirrhosis was obscure.

In addition, two patients with subacute yellow atrophy following infectious hepatitis were treated with albumin. Both of these patients had edema and ascites and were in a critical state when treatment was begun; the diagnosis was confirmed at autopsy.

In studies of the changes in caloric intake during the course of albumin therapy, careful records of the daily intake of protein, fat and carbohydrate were kept by trained dietitians. An effort was made to provide patients with an excess of food and calculations of the intake were made from the amounts offered and refused. Diets were kept high in protein; fats and carbohydrates were provided according to the taste of the individual. For nitrogen balance studies three-day collections of urine and feces were analyzed for total nitrogen by the macromethod of Kjeldahl; nitrogen intake was calculated from standard tables.

Salt was not restricted in the diet. Preliminary studies indicated that with such a

restriction the caloric intake fell, and, since therapy was chiefly directed toward increasing caloric intake, salt restriction was not continued.

Alterations in serum proteins under the influence of albumin were studied in detail in each case. Serum albumin and globulin were determined by the Howe method (10) with Kjeldahl digestion and nesslerization of the filtrate. Electrophoretic analyses were carried out on the serum and ascitic fluid in certain of the patients as a supplementary method of protein estimation. The effect on the serum proteins of changes in plasma volume associated with albumin therapy was carefully observed; plasma volume determinations were carried out by the method of Gibson and Evans (11).

In determining changes in liver function during the course of therapy the methods employed were identical with those presented in a previous study (12). Estimation of serum esterase activity was carried out in the Warburg manometric apparatus and the results expressed in terms of the amount of CO_2 released from bicarbonate buffer following the hydrolysis of acetyl choline (13). The normal range is 40–80 mm. CO_2 .

The antidiuretic activity of urines of six patients was measured by a bio-assay technic (14) in which male rats were hydrated and then injected intraperitoneally with a standard amount of dialyzed and concentrated human urine. The minutes required for the excretion of 50 per cent of the ingested water was taken as the assay time, which in normals ranges from 80–140 minutes.

Method of albumin administration. Albumin¹ was administered intravenously from a standard unit containing 25 gms. in 100 cc. of buffered diluent to which no preservative is added. This is the amount of albumin obtained from 500 cc. of plasma. The albumin used in 1945 contained 0.6–0.9 gms. of sodium per 100 cc., whereas in 1946 and 1947 the "salt-poor" product (0.3 gms.) was given. No marked difference between these two types in respect to diuretic effect could be ascertained.

Toxic Reaction to Albumin

Four of the 17 patients treated with albumin developed a febrile reaction on one or more occasions approximately four hours after administration. None of these reactions was considered serious although a temperature of 104° was encountered once.

Two patients (Nos. 2 and 5) developed esophageal hemorrhages during the period of albumin therapy. This occurred at a time when marked shifts in body fluids were taking place and diuresis was at its height. In neither case was the hemorrhage serious. Since patient No. 5 had bled previously on numerous occasions, it is possible that the hemorrhage was coincidental.

Cardiac complications were not encountered in any of the patients. However, another patient, not included in this series, who was given albumin for other purposes, developed pulmonary edema following 14 units of albumin from which she recovered with great difficulty.

¹ This material was prepared at the Harvard Fractionation Laboratories, Department of Physical Chemistry, from blood collected by the American Red Cross.

TABLE I
Summary of Clinical and Laboratory Data before and after Albumin Therapy in 17 Cases of Chronic Liver Disease

Case no.	Age	Sex	Glucose data before albumin	A g	Bili- re- vires- cin	Ex- tra- trans- fused	Immediate response to therapy	Duration of therapy	Final outcome of therapy
GROUP I	1	M	Albumin. Large liver, large spleen. Following all subsequent transfusions developed ascites that did not respond to therapy. General condition was fair, 4 percentoses.	0.5 3.6	15 0.6	15 0.6	Immediate diuresis with loss of all ascites. Improvement in appetite and general condition.	12 mos.	3 subsequent small hemorrhages following return to alcohol. Slight ascites after first hemorrhage. No improvement on dietary therapy over a period of 2 mos.
2	M	F	Rectal prolapse. General development of ascites and ascites despite morphine. Small liver, large spleen. 1 percentoses. 1 hemorrhage. Active for 3 mos.	0.5 3.6	15 0.6	15 0.6	Immediate diuresis with complete loss of ascites and ascites. Small esophageal hemorrhage at height of thorax and 2 trans-fusions given.	9 mos.	Continued improvement in appetite and general condition. Returned to full activity. Continued response of ascites. Marked gain in body weight.
3	M	F	Jaundice and esophageal hemorrhage over a period of 9 mos. History of cirrhosis obscure. Moderate ascites and ascites. Responded to 1%.	0.5 3.6	15 0.6	15 0.6	Initial diuresis with loss of ascites fluid. Ascites reappeared. Small hemorrhage. Increased appetite and strength.	15 mos.	No further ascites. Spleen appeared during second month. Ascites reappeared. Continued response to full activity. Serum albumin gradually decreased.
4	M	F	Jaundice and abdominal pain at age of 65. In- termittent jaundice period. 3 esophageal hemorrhages followed by generalized edema. Albumin given because of respiratory difficulty. By Active for 1 month.	0.5 3.6	15 0.6	15 0.6	Immediate respiratory relief. Gradual loss of ascites and ascites over period of 2 mos. Considerable general improvement.	2 mos.	Remained improved for 2 mos. and then devel- oped another hemorrhage with some 4 days. General recovery. Died following a spine- real anastomosis.
5	M	F	Infectious hepatitis at age of 13. Intermittent jaundice subsequently. Esophageal hemorrhage at 17. For 2 years large ascites and ascites in 4 mos. on dietary therapy. Small liver, large spleen. Active returned after 3rd hemorrhage.	0.5 3.6	15 0.6	15 0.6	Immediate and marked diuresis with complete loss of ascites. Albumin was rapidly brought to normal and remained normal until after esophageal anastomosis.	10 mos.	Normal serum albumin was obtained and no fur- ther ascites appeared for 3 mos. A high hem- orrhage resulted in ascites which responded to 1% albumin. After 2 months of therapy the phase occurred during this therapy but ascites continued to disappear. Spleen-thyroid anastomosis performed. Ascites reappeared. Ascites re- solved following operation disappeared after 15 additional units of albumin. Liver showed post hepatic cirrhosis.
GROUP II	6	M	Jaundice at age of 15. Alcoholism. Marked weight loss. Ascites and ascites for 8 mos. 30 percent- oses. Marked liver. 6 mos. trial on liver ex- tract, cod liver oil, high protein diet and vitamin- B ₁₂ . No improvement. General condition poor. Stage. Terminal state when albumin was begun.	0.5 3.6	15 0.6	15 0.6	Very little effect at first - a gradual in- crease in weight. Gradual loss of ascites and ascites. Albumin was rapidly brought to normal and remained normal until after esophageal anastomosis.	15 mos.	Almost complete recovery. Patient returned to normal weight. After first course of therapy, 15 additional units caused a also disappearance of this fluid. Weight increased. Patient gained weight. Re- sponded to 1% albumin. Ascites reappeared normally 1 yr. after albumin was started.
7	M	F	Alcoholism. Inactive for 1 yr. 4 percentoses. Hemorrhagic jaundice. 6 mos. on che- motherapy. General condition poor. Ascites from loss of ascites following hospitalization for 6 mos. Child was commencing ascites when albumin was begun.	0.5 3.6	15 0.6	15 0.6	Very gradual loss of both ascites and ascites despite normal serum albumin level for 2 mos. No further ascites. General condition improved. Albumin. Markedly improved appetite and general condition.	7 mos.	Complete loss of ascites and ascites. Gain in body weight. Return to normal activity. No further ascites. General condition improved. General condition.
8	M	F	Infectious hepatitis at age of 25 and again at 3 mos. prior to albumin therapy. Liver extract for 2 mos. Body weight loss continued. Re- solved.	0.5 3.6	15 0.6	15 0.6	After 2 mos. of therapy gradual loss of ascites and ascites. General condition im- proved. Appetite and general condition im- proved.	9 mos.	Returned to normal activity. Ascites returned after 2 mos. and general condition was poor. After 2 mos. of therapy general condition im- proved. General condition remained poor. Died suddenly because of intestinal obstruction un- derlying ascites. General condition deteriorated. Kramer's cirrhosis.
9	M	F	Diagnosis unknown. Intensive development of as- cites; present for 3 mos. 25 percentoses. Re- sponded to 1% albumin. General condition poor. Small liver, large spleen.	0.5 3.6	15 0.6	15 0.6	No diuretic response despite an approximate normal serum albumin level for 2 mos. No change in appetite or dietary intake.	6 mos.	No definite response to therapy. Percentoses continue to be necessary every 2 mos.

TABLE I—Continued

Case no.	Age	Sex	Clinical data before albumin				Immediate response to therapy	Duration of follow-up after therapy	Final outcome of therapy		Survival—relapse-free survival	Age at death	
			Illness—pre-therapeutic condition	Albumin—pre-therapeutic condition	Albumin—pre-therapeutic condition	Albumin—pre-therapeutic condition			A/G	Survival—relapse-free survival			
GROUP III													
10	10	M	Infectious hepatitis 18 mos. previously. Marked jaundice 6 mos. course of liver extract, choline, high protein diet and vitamins had no effect. High protein diet and vitamins had no effect. Marked weight loss. Terminal anasarca when albumin was begun. Very large liver, splenic aneurysm, bleeding gums, large spleen. Ascites for 4 mos.	1.3 4.7	30	12	27	Marked immediate diuretic effect with complete loss of edema and ascites. Feeling of well-being and improvement in appetite. No return to general activity for 3 mos. Bleeding gums persisted. Prothrombin time 30 sec.	11 mos.	No further paracenteses. Edema and ascites returned repeatedly and were easily controlled by additional albumin. 60 additional units were administered during 11 mos. Serum albumin and prothrombin time improved. Ascites regressed because of uncontrolled bleeding from prothrombin time 34 sec. Fibrinogen 130 mg-%. Autopsy showed post necrotic cirrhosis.	3.0 3.7	31	25
11	12	M	Gradual onset of jaundice, edema and ascites at age of 10. History of cirrhosis unknown. Generalized anasarca developed. 1 paracentesis. Marked weight loss. Large liver and spleen. Ascites for 2 mos.	1.0 3.7	31	4	4	Immediate diuresis with loss of edema and ascites. General improvement with increase in appetite and strength.	11 mos.	Edema without ascites appeared approximately 2 mos. later with immediate response to albumin. On 5 occasions albumin was administered with a rapid and complete diuresis each time and associated general improvement. Died of neurological complications suggestive of Wilson's disease. Liver showed post necrotic cirrhosis.	3.0 3.9	27	5.6
12	51	F	Severe diarrhea and jaundice at age of 10. Jaundice persisted. Edema and ascites developed 1 yr. later. Controlled for 8 mos. by l.v. liver extract and dietary therapy. Gradual decline and in terminal state when albumin was begun. Ascites for 3 mos.	1.0 2.7	30	11	24	Immediate diuresis with complete loss of edema and ascites. Slight general improvement.	2 mos.	Jaundice increased following therapy. Ascites reappeared. Died in cholelithiasis. Autopsy showed biliary cirrhosis.	2.6 2.5	34	20
13	35	F	Jaundice, fever and arthritis for 2 yrs. Marked edema and ascites for 3 mos. 2 mos. on l.v. liver extract. Albumin started because of terminal state. Large liver and spleen.	1.6 6.5	31	3.0	26	Marked diuresis after 8 units of albumin. Loss of all edema and ascites. Improvement in appetite and general condition.	28 mos.	Free of symptoms of liver disease for 2 yrs. despite persistent abnormality of liver function. Regained ability to form albumin for 2 yrs. Exploratory biopsy revealed that definite cirrhosis persisted. l.v. liver extract administration had no effect. Asclites reappeared 18 mos. ago which responded to 10 additional units of albumin.	3.0 4.9	36	0.8
14	16	F	Infectious hepatitis at age of 28. Complete recovery. 2nd attack 3 mos. before therapy followed by severe ascites and edema. 1 paracentesis. Large liver and spleen. Comotose condition when albumin was begun.	1.3 3.7	12	8	8	Diuresis with considerable loss of edema and ascites while in comotose state. No general improvement.	4 days	Patient died in com. Albumin did not alter course despite diuresis. Liver showed extensive yellow atrophy.	3.1 2.9	15	15
15	10	M	Infectious hepatitis with transition to edema and ascites 6 mos. later. Anasarca and terminal state when albumin was begun.	2.0 3.6	16	6	6	Diuresis with loss of edema and a large amount of ascites. No change in general condition.	10 days	Had despite diuresis. Death curve of illness not affected by albumin. Liver showed extensive yellow atrophy.	3.1 2.7	22	22
GROUP IV													
16	12	M	Infectious hepatitis at age of 29. Recovered. Inadrenaline jaundice associated with bilateral Mittlers disease 3 mos. before therapy. Large liver and spleen. No edema or ascites. No bleeding tendency.	2.0 4.9	22	6	6	Definite response in respect to appetite and general strength. Dietary intake increased.	6 mos.	Therapy raised serum albumin level and patient was able to sustain this by high caloric intake.	3.3 2.9	25	1.7
17	18	M	Infectious hepatitis 18 mos. before therapy. Persistent symptoms. Large liver and spleen. Anasarca upper. No edema or ascites.	2.0 5.2	27	1.3	6	No definite response to albumin. Dietary intake remained unchanged.	7 mos.	Serum albumin level remained higher than prior to therapy. Exploratory biopsy showed post necrotic cirrhosis.	3.3 5.0	26	1.6

CLINICAL RESULTS

In order to evaluate the results of albumin therapy, the 17 patients in this series have been divided into four main groups: I. Patients treated soon after the onset of ascites; II. Patients with long-standing ascites who required frequent paracenteses; III. Patients suffering from a marked albumin deficit associated with unusually severe liver damage; and IV. Patients with low serum albumin levels without edema or ascites. Table I summarizes the results of therapy in the entire series.

Group I. Five patients comprise the group of cases with early ascites. Two were the nutritional type of cirrhosis, two were cirrhosis of undetermined etiology, and one was cirrhosis after infectious hepatitis. Relatively small amounts of albumin produced a diuresis with loss of ascites and general improvement in each of the patients. Figure 1 illustrates the rapid loss of ascites in patient No. 5 in whom 10 units of albumin were sufficient to produce the desired diuretic effect.

Figure 2 illustrates the course of patient No. 3 who while on intravenous liver extract therapy showed some increase in the serum albumin level and some loss of the moderate edema and ascites that were present. However, the serum albumin subsequently fell and marked ascites developed. Following the administration of 9 units of albumin a diuresis occurred with loss of all edema and some ascites. Despite continued albumin therapy, further ascitic fluid accumulated and second paracentesis was necessary. After 21 units were administered, albumin administration was discontinued. Approximately two weeks later a second diuresis occurred. This patient has been followed for more than one year after the loss of ascites and has remained well. Minimal edema still develops during menstrual periods but ascites has never reappeared. The serum albumin level gradually declined following the period of therapy so that it may be assumed that a defect in the synthesis of this protein still exists but is less severe. The patient gained approximately 15 lbs. of body weight and returned to normal activity. This case illustrates the marked improvement that may occur despite persistent severe damage and impaired ability to synthesize normal amounts of albumin.

The remaining three patients of the group improved markedly on small doses of albumin. A rapid diuresis was obtained in each case. Esophageal hemorrhages have complicated the course of recovery of two of these patients, one of whom died following a spleno-renal anastomosis.

In each patient in this group albumin administration initiated a more rapid response than could have been expected with dietary and intravenous liver extract therapy. Four of the five patients were able to preserve their artificially elevated serum albumin levels by better synthesis of their own serum albumin.

Group II. This group includes four patients with a nutritional type of cirrhosis all of whom were severely ill with ascites that had been present con-

stantly for more than five months. Each patient had been given vigorous dietary and vitamin therapy for at least three months prior to the administration of albumin. Three had been started on intravenous liver extract but, because their condition was deteriorating alarmingly, it was believed that a

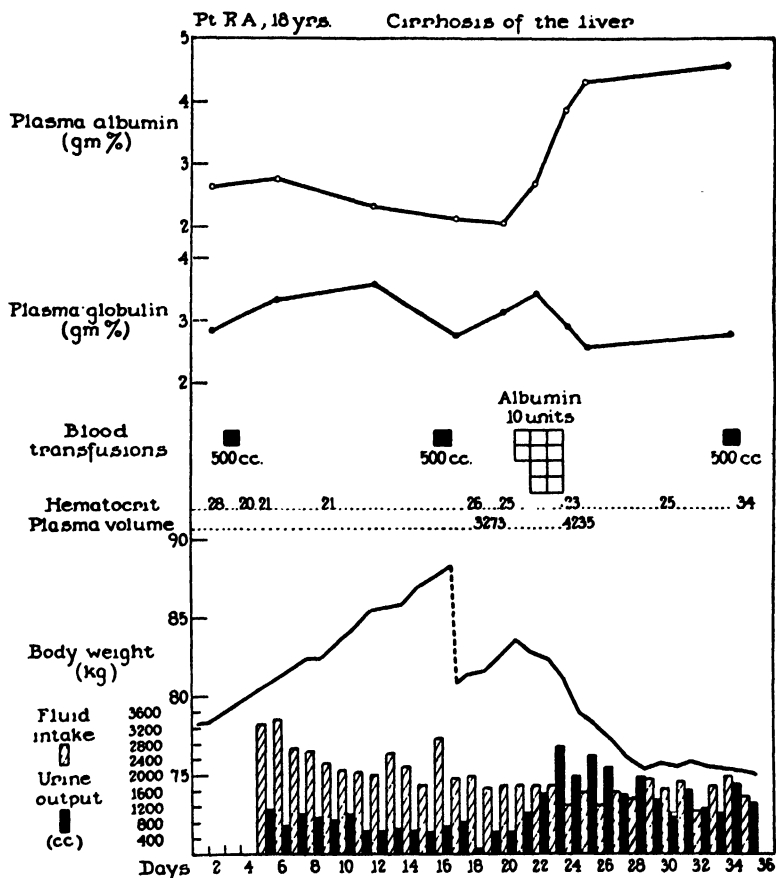


FIG. 1. Patient No. 5 (Group I) with early ascites following an esophageal hemorrhage. Rapid and complete diuresis following the administration of a small amount of albumin.

more rapidly acting form of therapy was necessary. This group, therefore, represents patients who would probably have succumbed to their disease despite the use of dietary and liver extract therapy.

Figure 3 illustrates the course of patient No. 6. The condition of this man grew progressively worse in the hospital while he was being given regular injections of liver extract. As may be seen from the weight curve, a continuous

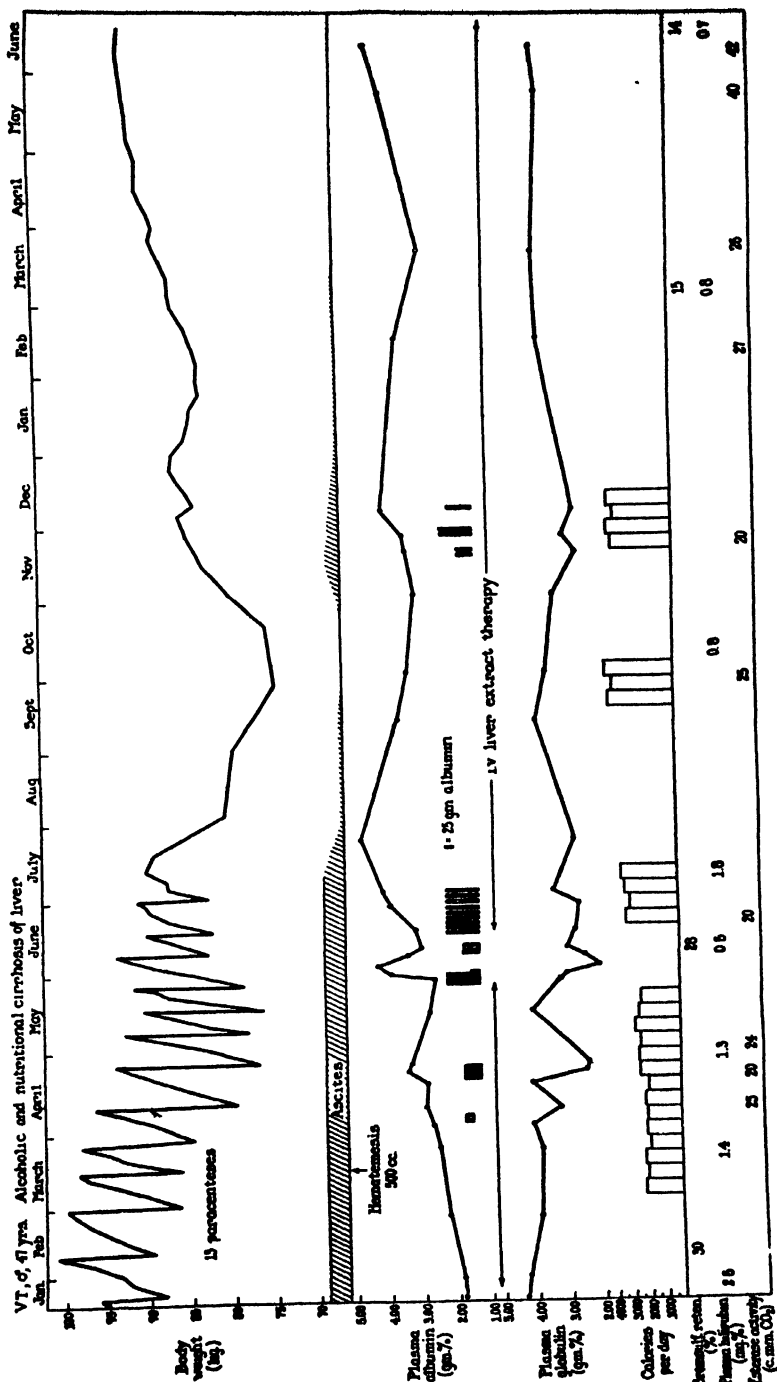


FIG. 3. Patient No. 6 (Group II) with ascites of eight months' duration requiring 30 paracenteses. Early rise of caloric intake following albumin. Markedly delayed diuresis following prolonged therapy with 80 units of albumin. Recurrence of ascites responding to 15 additional units. Return of ability to synthesize albumin one year after initial therapy.

decline in his basic weight followed each paracentesis. The administration of small amounts of albumin produced little effect. It was not until 4 units were given each day that the serum albumin level was raised to normal and a slight increase in urine volume occurred. The patient also noticed a feeling of well-being and the appearance of appetite that was reflected in an increase in caloric intake. Very little effect was noted, however, on the rate of accumulation of ascitic fluid until 4 units of albumin were administered every other day for more than two weeks. During this time a gradual slowing in the rate of fluid accumulation occurred although no acute diuresis was ever obtained. Therapy was finally stopped after 80 units of albumin had been administered despite the fact that considerable ascites persisted. Approximately two weeks after cessation of therapy a diuresis with loss of all ascites occurred. At the same time the serum albumin rose to the highest level that had been reached. Although his caloric intake was almost double the pre-treatment level, the patient failed to put on body weight. He remained free of fluid for three months but then slowly and despite continued intravenous liver extract therapy reaccumulated ascites until a paracentesis appeared necessary. Instead, 15 units of albumin were administered and a slight diuresis was obtained. Gradually over the next two months all ascitic fluid disappeared spontaneously, and the patient began to put on body weight. The improvement following the second course of albumin was more striking than after the first. The serum albumin which had gradually fallen following initial therapy was raised by the second course, but slowly fell once again. He gained approximately 30 lbs. of weight and returned to normal activity. Suddenly, six months after the last albumin treatment, the serum albumin rose spontaneously to normal indicating that the patient had finally regained the ability to synthesize normal amounts of serum albumin. His bromsulfalein retention fell from 30 per cent to 12 per cent over the period of 18 months that he was treated.

The three other patients in the group were treated with large amounts of albumin after failing to improve on other forms of therapy. All but one patient showed a gradual disappearance of ascites and marked clinical improvement. Ascites disappeared very slowly, usually several weeks after the patients had begun to feel improved and were eating better. In patient No. 8 moderate ascites recurred three months after therapy, although the patient continued to lead a normal life. Further albumin was not administered. This patient died suddenly because of an intestinal obstruction unrelated to the liver disease. Patient No. 9, who did not respond to albumin therapy, was unusual in that she had been accumulating approximately 20 liters of ascitic fluid every 14 days for nine months prior to albumin administration. The antidiuretic titer was found to be unusually high, approximately twice the normal value (Table III). Eighty units of albumin were administered intravenously over a period of three months without noticeable effect. Although

the serum albumin level was kept above 3.5 gms. per cent during this period, paracenteses continued to be necessary every 10 to 16 days.

Group III. Four patients with extremely severe cirrhosis and two patients with fatal subacute infectious hepatitis, all showing albumin levels below 2 gms. per cent, comprise this group. All demonstrated large amounts of edema associated with ascites. Laparotomy biopsy was performed on one patient, and autopsies were obtained on the five patients who died. None of the patients showed a typical Laennec's cirrhosis. The diagnosis was post-necrotic cirrhosis in three patients, biliary cirrhosis in one, and subacute yellow atrophy in two.

All patients demonstrated a diuretic response with disappearance of edema and ascites to an average of 16 units of albumin. Figure 4 illustrates the course of a typical case in this group. The patient (No. 10) was a 32 year old sailor who had had a typical attack of infectious hepatitis 18 months prior to therapy. Following a severe relapse of infectious hepatitis, symptoms and signs of liver damage persisted and he was followed for one year at the Hospital of the Rockefeller Institute. Following discharge from the Navy he attempted full time work, but his symptoms became more severe and he was readmitted to the Rockefeller Hospital with generalized anasarca and ascites. His condition was critical and during a month of dietary and vitamin therapy he required three paracenteses. Following the administration of 15 units of albumin a diuresis with loss of all ascites and edema occurred, and during this diuresis 12 additional units were administered. The serum albumin level was raised to normal and the general condition of the patient improved dramatically. His caloric intake increased from 2,000 to 3,000 calories per day and within two weeks he was able to return to full activity. It was evident that he suffered from a severe albumin deficit which when overcome resulted in marked improvement. Over a period of four months following the first course of therapy the serum albumin level gradually declined and the patient once again developed ascites and edema. The administration of 12 units of albumin produced another immediate diuresis. The patient lived for approximately one year after the onset of albumin therapy, during which time recurring edema and ascites were readily controlled by albumin administration on four occasions. Albumin, however, had no measurable effect in improving his liver function, and the serum bilirubin rose in the terminal four months from 12 mg. per cent to 25 mg. per cent. Synthesis of prothrombin and fibrinogen by the liver were markedly impaired, similar in degree to the albumin deficiency. However, these proteins were not available for replacement therapy, and the patient finally succumbed because of severe, uncontrollable bleeding from his gums and nose despite numerous transfusions with fresh blood. It was believed, however, that the patient's life had been lengthened considerably by albumin therapy.

CH, d, 32 yrs Cirrhosis of liver following infectious hepatitis

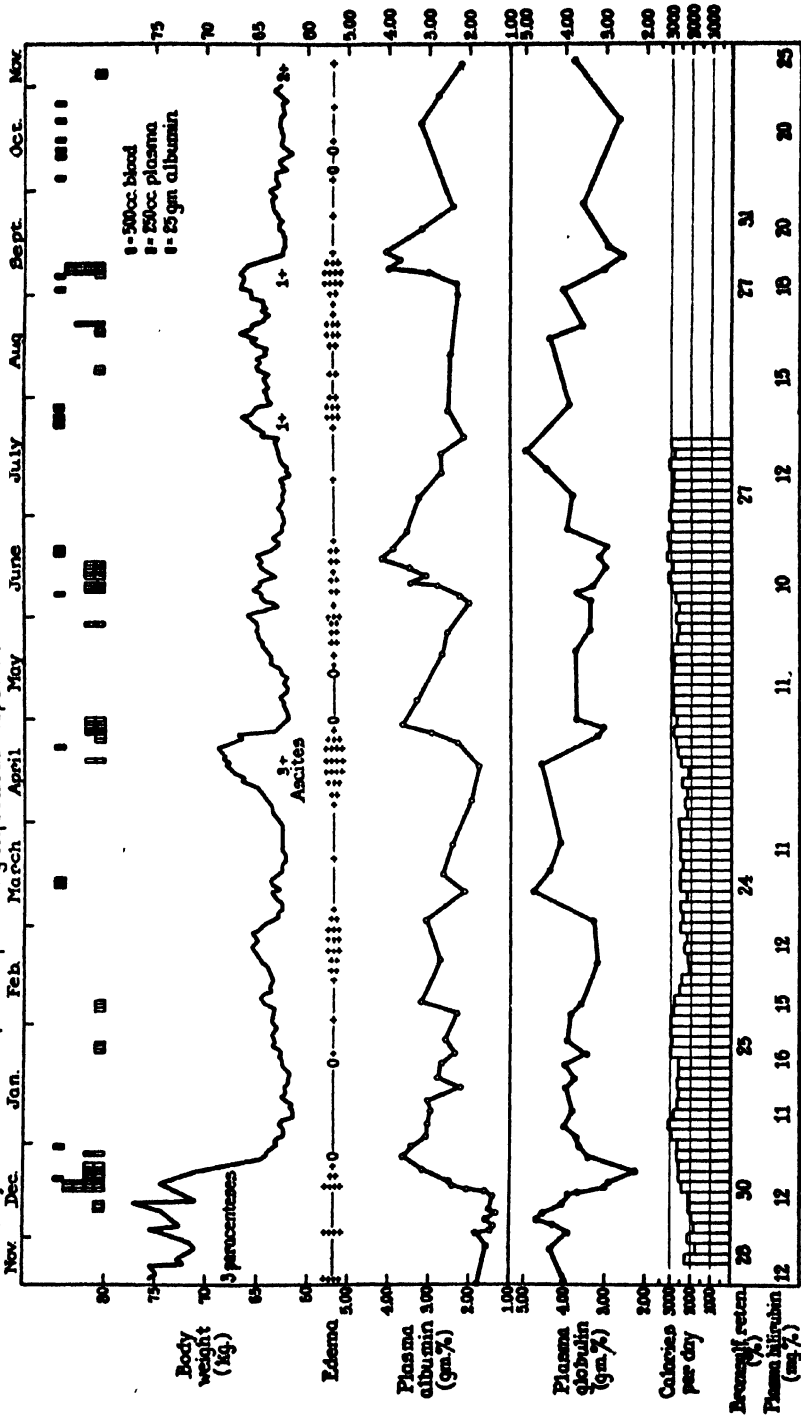


FIG. 4. Patient No. 10 (Group III) with generalized anasarca 18 months after infectious hepatitis. Rapid and complete loss of edema and ascites following 27 units of albumin. Control of recurrent ascites with additional albumin. Increased caloric intake with each course of albumin. Death due to uncontrollable bleeding tendency despite control of fluid balance.

Two other patients in the group (Nos. 11 and 12) appeared to have had their life span increased by albumin administration. Patient No. 11 received five separate courses of small amounts of albumin with loss of edema and ascites on each occasion. He eventually died during curare treatment for a neurological disorder resembling Wilson's disease. Patient No. 13, who also presented a terminal picture of generalized anasarca with a very low serum albumin, demonstrated a cirrhosis of undetermined etiology associated with severe arthritis. All edema and ascites disappeared rapidly following the use of intravenous albumin. This patient differed from the others in that she continued to preserve the normal serum albumin level brought about by albumin administration and has remained well leading a normal life for 25 months following therapy. Laparotomy biopsy two years after therapy showed an advanced cirrhosis characterized by dense fibrous tissue strands and large masses of regenerating liver cells. The gratifying response to albumin in this patient emphasizes the difficulty in predicting the therapeutic effect of albumin.

The two patients with subacute yellow atrophy (Nos. 14 and 15) showed a diuresis with loss of edema and ascites while in a semi-comatose state. Their downhill course was unaffected by the albumin therapy and both died. They are additional examples of cases that react readily with a diuretic response to relatively small amounts of albumin.

The factor of portal obstruction was not apparent clinically in this group of patients with severe liver disease. Water retention corresponded closely to the albumin deficit and could be controlled readily by albumin therapy. Although three of the four patients with cirrhosis eventually died, they were markedly improved temporarily by this form of therapy. This effect, together with the dramatic and prolonged improvement in patient No. 13, demonstrates the value of albumin in this type of liver disease.

Group IV. Two patients with post-hepatitis cirrhosis without edema and ascites were given albumin therapy in order to increase dietary intake prior to the expected onset of hepatic decompensation. Patient No. 16 showed a definite increase in caloric intake and was able to maintain his artificially raised albumin level. He gained weight and was able to return to normal activity. Patient No. 17 showed no response. The dietary intake remained poor, marked fatigue persisted, and weight loss continued. However, for seven months he maintained a serum albumin level higher than that prior to therapy.

Specific Effects of Albumin Therapy

The influence of injected albumin on the ability of the liver to resume normal synthesis of albumin is perhaps the most important consideration in evaluating its therapeutic effectiveness. The necessity for periodic injections of albumin

in the form of maintenance therapy rests with this question. Table II shows that four of 12 patients were able to form sufficient albumin to maintain an approximately normal level three months after the first course of therapy ended. One other patient (No. 6) regained this capacity following a second small course of therapy. Three patients experienced a rapid fall in serum albumin after artificial elevation of this level by a series of injections and demonstrated no improvement in their ability to synthesize albumin. The remainder maintained higher levels after treatment but did not show complete restoration to normal. It should be mentioned that the patients who developed low serum albumin levels and ascites several weeks after bleeding episodes were best able to maintain permanently normal levels following therapy.

TABLE II

Alterations in Plasma Albumin Level Following Albumin Therapy in 15 Patients with Cirrhosis of the Liver

Albumin level	No. of patients		
	Before therapy	Immediately after therapy	3 mos. after therapy*
3.5 gms. per cent or higher.....	0	15	4
3-3.5 gms. per cent.....	0	0	5
3 gms. per cent or lower.....	15	0	3

* Three patients are not included: two have not been followed for three months following the discontinuation of therapy and one died two months after therapy.

Nitrogen balance studies were carried out in four of the patients who received albumin. Figure 5 illustrates the results in a typical case. This patient was in slight negative nitrogen balance prior to therapy but then entered marked positive balance during the period of albumin administration. In the four patients studied there was greater than 90 per cent retention of injected albumin nitrogen; they lost ascites following albumin therapy and did not require further paracenteses. As a result, the continued loss of albumin through the peritoneal cavity was obviated. This was not true of the patients who required further paracenteses; they continued to lose albumin nitrogen. Further observations on the patient illustrated in Figure 5 showed that the patient remained in positive balance for at least one month after therapy was discontinued. Figure 5 also illustrates the marked difference between the effect of albumin on the nitrogen balance and of an equivalent amount of nitrogen in the form of casein hydrolysate.

A number of patients volunteered the information that they developed a feeling of well-being following the administration of albumin, despite the fact that no diuresis occurred. This was associated with a general increase in

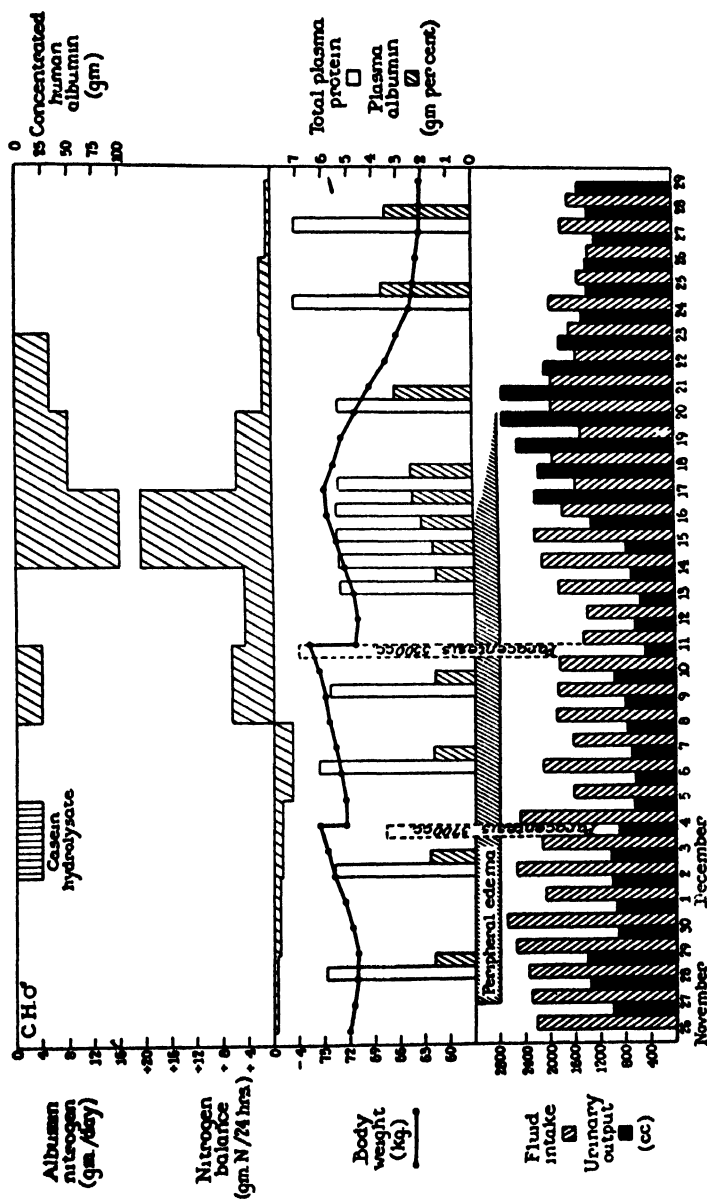


FIG. 5. Patient No. 10 (Group III). Persistent negative nitrogen balance with casein hydrolysate. Positive nitrogen balance with an equivalent amount of albumin nitrogen. Complete retention of albumin nitrogen.

appetite. To test the validity of these observations careful measurements were carried out on the caloric intake before and after albumin therapy in a patient who was free of ascites and showed only minimal edema. Figure 6

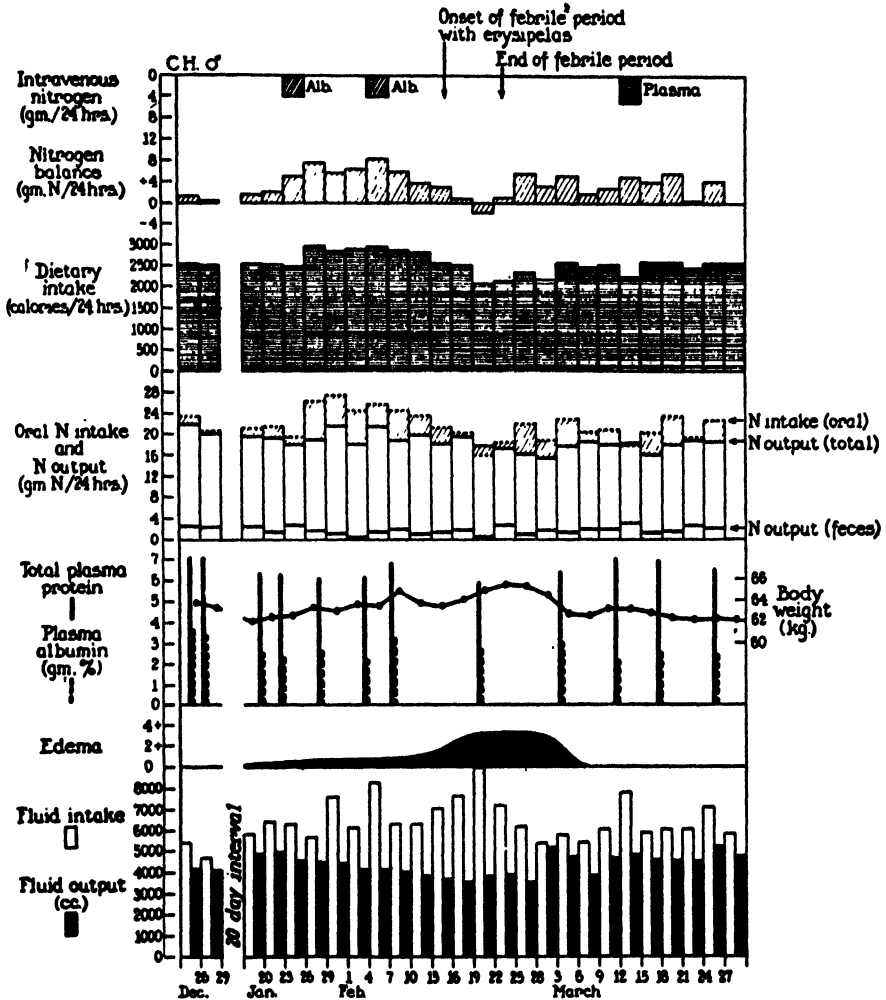


FIG. 6. Patient No. 10 (Group III). Rise in caloric intake and increased retention of dietary nitrogen following two courses of 3 units of albumin.

illustrates the definite rise in protein and total oral caloric intake following the administration of 3 units of albumin. Nitrogen balance determinations revealed an increased retention of dietary nitrogen in addition to the retention of the nitrogen of the albumin administered. The experiment was interrupted

by a short febrile period due to a skin infection. Subsequent administration of plasma did not affect the caloric intake or the nitrogen balance to a similar degree. This specific effect of albumin on the dietary intake was observed during four different periods of albumin therapy in the patient (Figure 4). In another patient (Figure 3) a similar rise in caloric intake during albumin therapy was observed considerably in advance of his diuresis.

Estimation of the degree of retention of bromsulfalein was carried out before and after albumin treatment in each of the patients. No improvement in this liver function test could be demonstrated within the first month after albumin therapy. However, in four of the patients who were followed for more than six months following therapy, definite improvement occurred. In patient No. 6 who exhibited no jaundice the bromsulfalein retention fell from 30 per cent to 12 per cent over a period of 16 months. No immediate improvement occurred in serum bilirubin, prothrombin time, total globulin, the thymol turbidity reaction, fibrinogen, and esterase activity following albumin therapy. However, with prolonged treatment for more than six months, changes occurred in these serum components which were in general parallel to the improvement in bromsulfalein retention.

DISCUSSION

The most apparent effect of albumin in patients with liver disease was that due to its osmotic properties. In nine of 15 patients with fluid retention who were treated, a rapid loss of edema and ascites occurred. Five others showed a delayed loss of ascites. In those patients whose diuretic response to albumin was immediate, a beneficial course of events was rapidly initiated; loss of protein into ascitic fluid ceased, dietary protein intake increased, and positive nitrogen balance resulted. Such treatment carried seriously ill patients through the early critical period of their disease until such time as dietary and liver extract therapy had an opportunity to take effect.

The amount of albumin needed to produce a loss of ascites was extremely variable, ranging from 4 to 80 units, and appeared to depend upon at least four factors. The first of these was the relative rôle of portal obstruction in the causation of fluid accumulation. Since there is no direct method of measuring portal pressure without laparotomy, it was necessary to evaluate this from clinical signs. Esophageal varices and other evidences of collateral circulation, a small hard liver and a large spleen were considered to be associated with portal hypertension. However, the most useful sign was the amount of edema relative to the amount of ascites. Those patients exhibiting marked ascites with little or no edema were considered to have a high degree of portal obstruction, while large amounts of edema in the presence of less impressive quantities of slowly forming ascites indicated that portal obstruction, if present, was of minor importance. Edematous patients with slight evidence of portal

obstruction responded more readily to therapy with albumin than did patients with severe portal hypertension. Patients with subacute yellow atrophy and cirrhosis following infectious hepatitis usually fell into this category. The plasma albumin levels of this group were extremely low, occasionally below 1.5 gms. per cent, suggesting that these patients suffered from a specific albumin deficit. Approximately 12 units, administered over a period of four days, usually produced a dramatic diuresis with loss of edema and ascites. On the other hand, patients with large amounts of ascites in the absence of generalized edema, in whom portal obstruction was considered to be a dominant factor, were generally very resistant to therapy. As much as 80 units of albumin were sometimes necessary before ascites disappeared. It should be emphasized, however, that a diuresis was produced in three out of four such patients with marked evidence of portal obstruction when the plasma albumin was kept at a level of 3.5 gms. per cent or higher for a sufficiently long period of time; the one refractory patient failed to respond to 80 units given over a three-month period despite normal serum albumin levels.

A second factor of importance in determining the amount of albumin required to produce a disappearance of ascites was the rapidity with which the patient accumulated fluid. Following the administration of albumin an equilibrium between blood and ascitic fluid albumin was established, and the A/G ratio in these two body compartments tended to remain equal. As a result, with the rise in albumin concentration of the serum, there was a rise in the concentration of albumin in the ascitic fluid. When the ascitic fluid collection was large and constantly reaccumulating, a sizeable drain on the the injected albumin would occur with each paracentesis. Small doses of albumin were ineffective under such circumstances.

The third factor and one which has not been clearly evaluated was the length of time that fluid accumulation had been present prior to albumin therapy. Those patients treated soon after they began to accumulate ascitic fluid responded readily to therapy in each case. The patients most resistant to the diuretic effect of albumin were those who had been accumulating fluid for long periods. The experience gained by treatment of the patients in Group I suggests that early ascites is due primarily to low serum albumin levels and that albumin therapy is a specific remedy at this time. In the patients of Group II with long-standing ascites, changes secondary to fluid accumulation have been added to the initial factor of hypoalbuminemia, rendering albumin treatment less effective. Investigations are now in progress regarding the rôle of alterations of peritoneal absorption in these patients.

The fourth factor influencing the amount of albumin necessary to produce a diuretic effect was the concentration of antidiuretic principle present in the urine of patients being treated. Preliminary observations indicated that those patients excreting large quantities of this substance required prolonged administration of albumin (Table III).

The ultimate effects of albumin therapy are certainly not all directly attributable to albumin alone. In certain patients this material simply initiated a curative process that was then sustained by increased caloric intake and liver extract therapy. A rise in caloric intake and increased retention of dietary nitrogen were demonstrated following albumin therapy in certain patients where the response could not be attributed to relief from the disabling effects of fluid. This effect of albumin was perhaps of even greater significance than its oncotic effects in initiating the process of recovery. In the three patients of Group II who eventually lost their ascites after prolonged albumin therapy, the effects of albumin on food intake and utilization may have been primarily responsible for the eventual disappearance of ascites. The fact

TABLE III

Comparison between the Titer of Antidiuretic Substance in the Urine and the Amount of Albumin Required and the Type of Response Produced by Albumin Therapy in Six Patients with Cirrhosis of the Liver

Case no.	Antidiuretic titer	Units of albumin required	Type of diuresis
	<i>minutes</i>		
5	140*	10	Immediate
2	140*	8	Immediate
11	120*	4	Immediate
7	300	24	Very gradual
9	240	80	None
6	160	80	Very gradual

* Normal.

that these patients demonstrated a delayed diuretic effect after albumin therapy raises the question whether supplemental nutritional and liver extract therapy which the patients were receiving continually might have been primarily responsible for improvement. However, against this possibility the following observations may be cited. First, during control periods on supplemental treatment alone these patients had failed to improve. Secondly, increased appetite and a general feeling of well-being were closely associated in time with albumin therapy. Finally, the most conclusive evidence for the primary rôle of albumin was obtained in those patients (Nos. 6 and 13) who developed a recurrence of ascites several months after the first period of therapy and in whom another small course of albumin caused improvement and loss of ascites for the second time.

SUMMARY

1. The results of serum albumin therapy in 17 patients with severe liver disease are presented.

2. Fourteen out of 15 patients with ascites lost their fluid following therapy.
3. The amount of albumin necessary to produce such an effect was variable ranging from 4 to 80 units.
4. Patients with marked evidence of portal obstruction, a high antidiuretic titer in the urine or long-standing ascites proved very resistant to therapy.
5. Patients with particularly severe liver disease and very low plasma albumin levels following infectious hepatitis responded most readily to therapy. Cases of alcoholic and nutritional cirrhosis with a short period of ascites also responded to relatively small doses of albumin.
6. Permanently beneficial results were obtained in six of seven patients with the nutritional type of cirrhosis. Two of these patients required a second short course of therapy. Patients with post-necrotic cirrhosis after infectious hepatitis and biliary cirrhosis showed only a temporary response and four of these patients eventually died.
7. Evidence was presented for certain specific effects of serum albumin on dietary intake and nitrogen balance which may be of greater importance in the results obtained than its osmotic properties.

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STUDIES ON NEWCASTLE DISEASE VIRUS

I. AN EVALUATION OF THE METHOD OF TITRATION

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Electron microscope studies of purified preparations of the virus of Newcastle disease have shown that this virus has a filamentous to sperm-like form when dried from saline suspensions (1, 2). If, however, the virus is suspended in water and then dried from this suspension, it has a roughly spherical form, which corresponds to its appearance in the original allantoic fluid (3). An apparent conversion from the spherical form in water to the filamentous form has been brought about by the addition of saline to make a 0.07 to 0.15 M solution (3). The change in shape revealed by electron microscopy was brought about without any detectable change in activity of the suspension. Two questions must be answered before these observations can be assigned any basic significance. (a) Could the lack of a detectable change in activity be merely due to inadequacies in the methods of measuring activity? In other words, might we destroy 95 per cent of the virus by the procedure which changes its shape, and not detect this loss of active virus particles? (b) Does this change in shape on transfer to saline represent a change which occurs within the solution, or is it merely the result of drying from different solvents? Some experimental evidence against this latter possibility was previously mentioned (3), but more work needs to be done.

We attempt in this paper to evaluate the methods of determining activity (embryo infectivity) and conclude that the changes in shape of the virus cannot be accounted for on the basis of disintegration.

The determination of the 50 per cent mortality or infectivity end-point (4) by means of chick embryos has been in use for a number of years, but very few reports have been made on the accuracy of the method used. Indeed, there is no statistical study available which attempts to estimate the expected probable error with a given number of animals and a given titration end-point slope.

In a limited number of duplicate titrations of Eastern equine encephalomyelitis, a variation of as much as 0.6 log was found between two duplicate titrations (5), using five embryos in each of three dilutions. In a more extensive evaluation of the 50 per cent infectivity measurements for influenza virus in the embryo, Knight (6) found that the chances were 19 out of 20 that differences in end-points of 0.62 logarithmic units were significant.¹ This means that more than 75 per cent of the infectiousness

¹ Five embryos in each dilution.

of a preparation must be destroyed before the odds are as much as 20 to 1 that any decrease at all can be stated to have occurred. The accuracy is, of course, increased by increasing the number of embryos used.

The consistency of results obtained by Sigurdsson (7) when measuring the rate of multiplication of vesicular stomatitis virus in 10-day-old embryos would indicate a roughly similar accuracy for this virus when titered on 7-day-old embryos. Similar consistent results have been obtained in measuring the concentration of equine encephalomyelitis after 23 hours' inoculation (5).

It is, however, at present impossible to predict the accuracy of the method for a new virus infection since the sharpness of the end-point in itself would influence the accuracy of the determination. Quantitative determinations of the sharpness of the end-point, or end-point titration curves are available for only a few animal viruses (8-11) and those not on the embryo.

In a later paper in this series it is shown that infection and death of the embryo are produced by a very few particles of Newcastle virus (12). By use of the theory of random distribution of particles as outlined in the Poisson theory, it is possible to draw a theoretical curve for the expected number of infectious units needed to produce infection. This may be illustrated by two extremes. If one unit is capable of producing infection and a solution containing just enough material to produce infection is diluted $\frac{1}{10}$, then the inoculation to ten times as many embryos with this dilution produces the same total number of infections. If, however, it is necessary to add 1000 infectious units to an embryo to initiate an infection, and a drop of a given suspension contains this much, an equal portion of $\frac{1}{10}$ dilution would rarely have sufficient particles in it to initiate infection. Therefore, the next tenfold dilution would almost always be negative.

These considerations are raised here because they have direct bearing on the accuracy of the method. Fig. 1 shows that the points obtained with small dilutions of the virus best fit a one particle (infectious unit) curve. This does not prove that infection is produced by one unit, but is of practical importance in demonstrating the difficulty in using an end-point other than the 50 per cent end-point.

Materials and Methods

Virus.—The strains of Newcastle disease virus used in this series of studies are as follows: (1) Strain B isolated from a natural outbreak by Dr. F. R. Beaudette of the New Jersey Experiment Station; (2) strain W isolated by us from a natural outbreak of the disease in a flock of chickens in Bound Brook, New Jersey; (3) strain Np isolated by Dr. J. R. Beach in California; (4) Cg179, a laboratory passage virulent strain also isolated by Dr. Beach in California. This strain differs from the others in that it is able to kill 3-month-old chickens in high dilutions following intramuscular inoculation and agglutinates red blood cells poorly. Neutralization tests on embryos with strain W against classical sera furnished by Dr. Brandley demonstrated the immunological similarity. Intramuscular injection of chickens with strain B immunized chickens to Cg179, and agglutination inhibition tests with convalescent sera

from recovered birds inoculated with Cg179 showed the cross-relationship. All four strains are characterized by a filamentous shape when dried from salt solutions (1), produce a high titer of infectivity in the allantoic fluid of embryos, kill the embryo in 2 to 3 days, and often produce a characteristic hemorrhagic pattern which involves the brain and feather follicles.

Method of Titration.—Suspensions of virus were titered after preliminary low speed centrifugation at 5,000 R.P.M. for 5 minutes. Serial tenfold dilutions were made in tubes containing 4.5 cc. of cold buffered saline which had been kept at refrigerator temperature just prior to the titration. Immersion in ice water was omitted because of the stability of this virus. Ten to 12-day-old embryonated eggs opened by making a window in the side of the shell were inoculated with 1 drop of the dilute suspension. All eggs were obtained from the

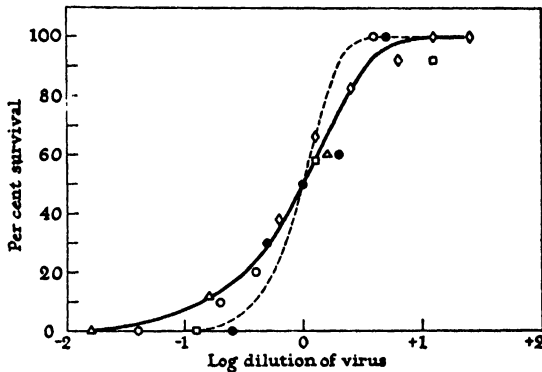


FIG. 1. End-point titration curve of Newcastle virus. Points on this curve were obtained by inoculation of ten embryos at each dilution. A 50 per cent end-point was calculated. The actual per cent of embryos surviving at a given log dilution above or below this calculated end-point is plotted against the log difference between the calculated 50 per cent end-point and the dilution inoculated. Symbols of the same kind represent one experiment. We have combined the results of five experiments to obtain the data. The solid curve is the theoretical curve for infections produced by one infectious unit. The dotted curve is for three units.

Rockefeller Institute stock which has been repeatedly found free of Newcastle disease as shown by the lack of immunity demonstrated by red cell agglutination inhibition tests and inoculation of virulent strains of the virus.

Embryos were usually incubated at 35° after inoculation (see below) and the occasional embryo dying within the first 12 to 18 hours was considered as killed by trauma and was discarded. With dilute suspensions of virus, death of the embryo begins about 2½ days after inoculation and may continue through 3 or 4 days. Incubation at higher temperatures produces more rapid multiplication of the virus and consequently a more rapid death. All surviving embryos were kept for 6 days after inoculation before discarding.

Smears were made as routine from dead embryos for microscopic examination to eliminate the occasional death occurring from accidental contamination. Previous work with gonococcus prophylaxis in embryos (13) has shown that this method picks up the great majority of such infections. However in critical experiments, blood plate cultures were also made. Since the virus of Newcastle disease agglutinates red cells, we usually checked the ability of the allantoic fluid from dead embryos used in a titration to do this. There are, however, several difficulties involved. First, concentrated suspensions of unpurified virus often show a prozone effect—possibly due to an inhibitor in the allantoic fluid. Therefore, it is necessary

to check two or three tenfold dilutions of the allantoic fluid before stating that it is negative. Secondly, different strains vary in their ability to agglutinate. Strain Cg179, our virulent laboratory strain, is a poor agglutinator producing irregular agglutination of chicken red cells only up to dilutions of 1/100 or so. Thirdly, embryos inoculated on the chorioallantoic membrane do not always develop sufficient virus in the allantoic fluid to agglutinate red cells. Thus, we cannot consider deaths which occurred at the right time and in the absence of bacteria, as not being due to Newcastle virus if they did not show red cell agglutination. However, several of the five embryos in a dilution would usually, if they died, have sufficient virus to agglutinate chicken red cells, and we could therefore conclude that the virus was present in that particular dilution.

It is well to emphasize that similar accuracy to that obtained here cannot be expected if the source of embryos is not from a clean flock of chickens, and if the above considerations are not taken into account. Particular care should be taken to demonstrate that deaths from other causes not following inoculation are not occurring in the eggs between the 10th

TABLE I
Duplicate Titrations of Various Suspensions
50 per cent end-point mortality. 3 dilutions—5 embryos each dilution

Virus	Duplicate titers		
	Titer 1	Titer 2	Difference
B—allantoic fluid.....	10 ^{-7.4}	10 ^{-7.2}	-0.2
B— " ".....	10 ^{-8.7}	10 ^{-8.6}	-0.1
Cg179—" ".....	10 ^{-8.5}	10 ^{-8.4}	-0.1
Cg179—" ".....	10 ^{-7.8}	10 ^{-8.0}	+0.2
B— " ".....	10 ^{-8.7}	10 ^{-8.5}	-0.2
B—embryo suspension.....	10 ^{-8.2}	10 ^{-5.8}	-0.4
B—purified preparation.....	10 ^{-7.6}	10 ^{-7.8}	-0.2
Same titred in water.....		10 ^{-7.8}	

and 18th days of incubation. This may be a common source of error when using eggs bought on the open market.

RESULTS

Table I presents the results of duplicate titrations on different preparations carried out during the course of our investigation. None of these seven tests differed from the duplicate by more than 0.4 log. We therefore believe that a difference of 0.6 log is probably significant and of 1.0 log almost certainly significant. This would mean that we probably could detect a loss of 75 per cent of activity and almost certainly a loss of 90 per cent activity. A variety of other factors might be of significance in titering Newcastle virus in the embryo and we here present data bearing on some of them.

Temperature.—It has been shown for a number of viruses that the optimum temperature range is fairly narrow. For influenza it is about 35–36°C. This suggests that titrations might better be carried out at that optimum. How-

ever, the optimum temperature range for growth of Newcastle virus seems to be much greater (14). The effect of different temperatures of incubation after inoculation of the embryos with the definitive dilutions was studied by performing titration as usual, but ten instead of five embryos were inoculated at each dilution. One-half of the embryos in each dilution were incubated at one temperature and the other half at another. The 50 per cent end-points were calculated separately and are compared in Table II.

TABLE II

Effect of Temperature of Incubation on Titer Obtained from Same Preparation of Virus

Age of embryo used	Temperature of incubation	
	35°C.	39°C.
10 days.....	$10^{-6.5}$	$10^{-6.3}$
14 days.....	$10^{-6.5}$	$10^{-6.7}$

TABLE III

Effect of Route of Inoculation on Titration Results

Virus	On chorioal- lantoic membrane	In allantoic sac	In amniotic sac	In yolk
Cg179.....	10 ^{-8.2}	10 ^{-7.8}	10 ^{-8.2}	10 ^{-9.0} 10 ^{-9.3} 10 ^{-8.8}
Cg179 6-day-old fluid.....	10 ^{-7.8}	10 ^{-8.2}		
B strain suspension embryo.....	10 ^{-6.7}	10 ^{-7.2}		
B strain allantoic fluid.....	10 ^{-9.5}	10 ^{-9.5}		
B strain concentrated.....	10 ^{-9.2}			
B strain 46 hr. growth.....	10 ^{-8.5}			
Cg179 stock.....	10 ^{-7.5}			

Route of Inoculation.—With different strains of mumps it is necessary to introduce the inoculum by different routes to get the highest degree of takes (15). The virus of swine influenza is likely to kill the embryo if injected into the allantoic sac (16), but if inoculated by itself on the chorioallantoic membrane, relatively few of the embryos die (17); many throw off the infection and survive. It might therefore be expected that the route of inoculation would play some rôle in determining the 50 per cent end-point. A number of tests were made, again by inoculating the same dilution fluid by different routes into several series of embryos.

Table III shows the results from a series of such tests.

The inoculation on the chorioallantoic membrane was performed by first lowering the membrane through an artificial window in the side, then dropping 1 drop of the dilution tested on the membrane, and sealing with scotch tape. Allantoic inoculation was usually performed

by injection of about 0.05 cc. through a small hole in the otherwise intact egg with a short hypodermic needle. Amniotic inoculation was by direct inoculation with the visualization of the amniotic sac through an artificial window in the side. A pair of fine forceps held the edge of the amniotic membrane. Yolk sac inoculation was by a long needle (1½ inches) inserted through the blunt end of the egg.

Table III shows that there is no consistent difference between the methods of inoculation and that they all yield comparable results. This is in agreement with other recent reports (18). We continue however to prefer for this virus the inoculation on the chorioallantoic membrane because we believe that this method allows a more careful check of accidental deaths and gives slightly more consistent results.

Effect of Age of Embryo.—The effect of the age of the embryo used in measuring virus activity was studied by inoculating the same dilutions on two sets of

TABLE IV
Influence of Age of Embryo Used in Titration on the Calculated 50 Per Cent End-Point

Experiment No.	Age, days					
	10	11	13	14	15	16
1	10 ^{-8.3}				10 ^{-8.3}	
2	10 ^{-6.5}			10 ^{-6.5}		
3		10 ^{-7.8}		10 ^{-7.5}		
4	10 ^{-9.2}		10 ^{-8.8}			10 ^{-7.8}

embryos (chorioallantoic membrane) of different ages. The results of this are shown in Table IV. All inoculations included in this table were with strain B.

Three of the four experiments failed to show any effect of the age of the inoculated embryo. The fourth did show a probably significant effect of age. We, therefore, prefer 10- or 11-day embryos for titrations, but 12-day embryos may also be used, and when used in these experiments their use has been so specified.

Effect of Red Blood Cells.—Several of the viruses which agglutinate red blood cells are absorbed on the chick red cells in large amounts. It might, therefore, be expected that accidental contamination of the fluid with red blood cells during harvesting would considerably affect the titer obtained. However, actual tests indicate that at room temperature a minimum amount of virus is absorbed. In order to give a maximum chance for absorption the following experiments were done with the B strain which is a good agglutinator. The allantoic fluid virus was diluted to $\frac{1}{100}$ and to this a sufficient amount of red blood cells to make a 1 per cent suspension was added. Since agglutination is better at ice box temperature (19), the virus suspension with red cells was placed in the refrigerator for varying periods of time. The chicken red cells

were then spun down and the supernatant fluid titered for virus activity by inoculation of serial tenfold dilutions. Controls were similarly treated except for the absence of red blood cells. The results are shown in Table V.

There is a consistent reduction in the embryo infectivity. This reduction is only occasionally statistically significant. These results would agree with those of Florman in indicating absorption of the virus on the red cell, but indicate clearly that a few red cells gaining entrance into the allantoic fluid when harvesting are not an important source of error.

TABLE V
*Effect of Addition of 1 Per Cent Chicken Red Blood Cell Suspension
to 1/100 Dilution of Virus*

Allantoic fluid virus	Time after red cells added, min.						Control
	Immedi- ately	7	20	30	45	60	
Diluted 1/100.....					10 ^{-8.5}		10 ^{-8.7}
" 1/100.....				10 ^{-7.0}			10 ^{-7.5}
Purified by centrifuga- tion. Dilution 1/10..	10 ^{-9.0}	10 ^{-8.5}	10 ^{-8.8}			10 ^{-9.2}	10 ^{-9.2}

SUMMARY

The application of the 50 per cent embryo mortality to a study of the virus of Newcastle is described. It has been evaluated by a series of duplicate titrations of the same sample of virus. In seven such titrations the largest difference between the two was 10^{-0.4}. It is therefore believed that a difference of 0.6 log is probably significant and of 1.0 log almost certainly significant. This would mean that we can almost certainly detect a loss of 90 per cent of activity.

Neither temperature of incubation nor route of inoculation in the test embryos had consistent effect on the measurement of virus activity. The effect of increasing age of the incubated embryo, from 10 days up to 16 days, is slight and inconsistent. The addition of chicken red blood cells to a dilution of virus may lower the titer of the preparation, but the change is not sufficient to be of importance in the routine handling of the virus.

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STUDIES ON NEWCASTLE DISEASE VIRUS

II. BEHAVIOR OF THE VIRUS IN THE EMBRYO

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A study of the growth and behavior of an individual virus in the developing chick embryo has two important aspects. The biologist who is endeavoring to understand the comparative pathology of various experimental infections must have detailed information on this point. The chemist or commercial immunologist who uses the egg more as a test tube needs to know under what conditions he can obtain the maximum titer of virus. Although a large proportion of the same basic information is useful to both groups, it is more from the point of view of comparative pathology that these data on the virus of Newcastle disease have been collected and will be analyzed.

Methods

The general methods used are those reported in the first paper in this series. Most of the measurements have been made by titrations of embryo infectivity, since it is not certain that the activity of the virus to agglutinate red cells is inseparable from such infectivity.

Distribution of the Virus in the Embryo

Burnet (1) demonstrated in the first studies of embryo infections with Newcastle virus that the final concentration in the allantoic fluid was higher if the embryo was inoculated in the allantoic sac, and higher in the amniotic fluid if this sac was inoculated. Since then it has clearly been shown that route of inoculation plays a large rôle in the amount of virus obtained from different parts of the embryo (2). Since this is so, any comparison of the distribution of the different viruses in the embryo must take into account the route of inoculation. Our comparison presented in Table I is useful in demonstrating that, following either inoculation of the chorioallantoic membrane or into the allantoic sac, there is about 100 times as much virus in the allantoic fluid as is present in the embryo. There is however a high concentration of virus in the embryo following such inoculations. Newcastle virus then may be placed between the encephalitis groups of viruses and the influenza group. Following membrane inoculation, the viruses of Eastern and Western encephalitis (3) and of Venezuelan encephalomyelitis (4) attain the highest titer in the embryo and chorio-

allantoic membrane. West Nile virus localizes in membrane and embryo regardless of route of inoculation (5). Japanese B encephalitis also attains a higher titer in the embryo when inoculated by yolk sac (6). Newcastle virus is like these encephalitic viruses in that it kills the embryo and has a high titer in the embryo when inoculated by membrane or allantoically. However, the concentration is not as high as that of the allantoic fluid, and Newcastle virus thus corresponds to the influenza group of viruses which, following inoculation into the allantoic sac, multiply rapidly and attain their highest concentration there. To summarize, we may say that Newcastle virus resembles the encephalitis group in its ability to spread throughout the developing egg and to attain a high concentration in the embryo, but that it resembles influenza virus in its high concentration within the allantoic fluid before death.

The distribution of a virus cannot be considered separately from the growth rate. Two factors known to have an effect on the growth rate in other embryo

TABLE I
Distribution of Newcastle Virus in 11-Day-Old Developing Eggs

Route of inoculation	Allantoic fluid	Amniotic fluid	10 per cent embryo
Allantoic sac.....	$10^{-9.5}$		$10^{-7.2}$
" ".....	$10^{-9.2}$	$10^{-8.2}$	
" ".....	$10^{-9.5}$		$10^{-6.7}$
Membrane.....	$10^{-7.8}$	$10^{-5.5}$	$10^{-6.2}$
" ".....	$10^{-9.5}$		$10^{-6.7}$

infections, (1) size of inoculum and (2) temperature of inoculation, have been studied in this disease.

It has been shown that inoculation of concentrated suspensions of influenza virus, of either A or B strain, will produce a lower final titer of virus in the allantoic fluid than will the inoculation of a more dilute suspension. This is apparently part of the large problem of interference by dead virus (7). Such an effect has not been found in the case of Japanese B encephalitis (6). Present studies on Newcastle virus fail to show any effect of the size of inoculum on the amount of virus finally obtained (Table II). Indeed the larger inoculum produced a larger amount of virus earlier in the course of the infection, and in consequence killed the embryo earlier. Following minimal inocula the same high titer was obtained later and death was delayed.

Effect of Temperature on Rate of Growth

The effect of temperature of incubation on the growth of a number of viruses has been recently summarized (8) and it has been pointed out that "chick embryos are more susceptible when incubated at 35 to 37°C. than at 39°C."

This generalization does not hold for Newcastle virus as shown in Table III. Indeed such an effect is not to be expected, for this virus must multiply in a

TABLE II
Effect of Size of Inoculum on Yield of Virus Incubated at 37° C.

Experiment No.	Time of incubation	50 per cent end-point of allantoic fluid			
		Inoculum			
		10 ⁻⁹	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶
	<i>hrs.</i>				
1	6	10 ^{-5.4}	10 ^{-3.4}	10 ^{-2.4}	
	24	10 ^{-8.3}	10 ^{-8.0}	10 ^{-7.3}	
	43	10 ^{-9.3}	10 ^{-9.3}	10 ^{-8.0}	
2	11½			10 ^{-4.8}	10 ^{-1.8}
	22			10 ^{-8.0}	10 ^{-5.3}
	46			10 ^{-8.5}	10 ^{-9.2}
	71				10 ^{-9.5}

TABLE III
Effect of Temperature of Incubation on the Yield of Newcastle Virus from the Allantoic Fluid of 11-Day-Old Embryos Inoculated into the Allantoic Sac

Experiment No.	Dilution of virus inoculated	Time of incubation	35°C.		37°C.		39°C.		40°C.		41°C.	
			Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
		<i>hrs.</i>										
1	10 ⁻²	48	10 ^{-8.5}	1/800	10 ^{-9.5}	1/800	10 ^{-9.7}	1/1600				
2	10 ⁻²	40	10 ^{-8.7}	1/6400			10 ^{-8.7}	1/6400			10 ^{-9.4}	1/6400
3	10 ⁻¹	41	10 ^{-8.5}		10 ^{-8.8}				10 ^{-8.7}			
4	10 ⁻³	48	10 ^{-8.5}								10 ^{-9.4}	
5	10 ⁻⁴	8½	10 ^{-3.4}						10 ^{-5.0}			
		18½	10 ^{-6.5}						10 ^{-8.0}			
		26	10 ^{-7.6}						10 ^{-8.6}			

host (chicken) which normally has a rectal temperature of 40 to 41°C. Furthermore, studies of the influence of temperature on the yield of virus obtained in the influenza-encephalitis group of viruses have usually not been related to time. Encephalitis virus will grow better at 37° than at 42°C., but initial growth rates seem to be about the same (3). Vesicular stomatitis grows better at 35–36°C.

than at 39–40°C., but again initial growth rates in both 7- and 10-day embryos are about the same (9). The virus of influenza B does better at 35°C. than at either 37° or 39°C., but the initial growth rates at 35° and 37°C. are very similar (10). This suggests that the relation of temperature of incubation to the growth of viruses in the embryo is not a simple problem of optimum temperature of incubation but that complicated host-parasite relations may play a large rôle (8).

Further data on the effect of temperature of incubation on the growth rate of Newcastle virus are presented in Chart 1. It may be noted that growth at

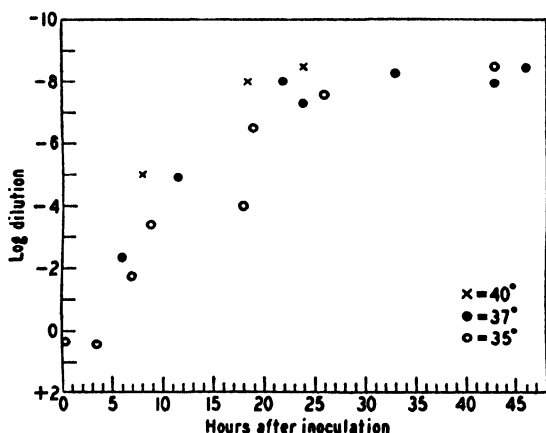


CHART 1. The relation of temperature of incubation to the growth rate of Newcastle virus in the allantoic sac of 11-day-old chicken embryos. All embryos were inoculated with a 10^{-4} dilution of freshly harvested allantoic fluid. Each point represents the titer obtained by pooling several embryos. The chart is a composite, but individual experiments (Table III, Experiment 5) demonstrate clearly the effect of higher temperatures. The titers below a log dilution 10^{-6} were obtained by inoculating 10 drops of the undiluted fluid to be tested (10^{+1} dilution).

40°C. is more rapid than at 37°C. and this in general more rapid than at 35°C., but that the final titer is about the same (Table III). As with other embryo infections (3, 4, 9–11), and some other animal virus infections initial growth through a logarithmic (12, 13) phase occurs without pathological changes, and the highest titer of virus may be obtained before pathological changes set in.

In the succeeding paper certain attempts to separate the red cell-agglutinating activity of Newcastle virus from the embryo infectivity are presented. It may be worth giving here a detailed record of the rise in red cell titer in the allantoic fluid and its relation to the infectious titer of the fluid. Henle and Henle (7) have shown in the case of influenza infections that correlations between the content of active virus in allantoic fluid and hemagglutinin titer may exist only during the stage of rapid increase of the active virus (logarithmic

phase of growth) but not after the active virus titer has reached its peak and started to decrease. Presumably this is related to loss of embryo infectivity (death) without loss of hemagglutinin activity. In comparing these results with those for the Newcastle virus we must remember that embryos infected with influenza virus frequently live for some days after inoculation and after the maximum titer of virus is obtained in the allantoic fluid. This is not equally true of Newcastle virus, for death occurs sooner after its maximum titer has been obtained. It is also more stable than influenza virus. Therefore, the relation of embryo infectivity to hemagglutinin activity after death would not be dependent upon peculiar host-parasite relations but merely would mirror the persistence of the more stable hemagglutinin characteristics as contrasted with embryo infectivity. Hence we cannot present any significant data on Newcastle virus after 48 hours (with dilute inoculum 72 hours).

Table IV presents a summary of our data on the relation of these two characteristics during the logarithmic phase of growth, following the inoculation of different concentrations of virus. When the known error in the method of determining embryo infectivity is considered (only three embryos were used in each dilution in this test) it is seen that we can conclude only that there is a concomitant increase in red cell-agglutinating activity and in embryo infectivity, and that the geometric rate of increase of hemagglutinins ceases at about the same time as does the embryo infectivity (20 to 25 hours) (Chart 1). Thus there is in our study no evidence of a separation of the two functions.

The question of a latent period before the logarithmic phase of growth begins was considered. The data in Chart 1 are inadequate to a decision. The embryos were inoculated with $\frac{1}{30}$ cc. of a 10^{-4} dilution of allantoic fluid, and this means that each embryo received about 50,000 LD₅₀ doses. This was diluted by a factor of $\frac{1}{30}$ cc. in 50 cc. (volume of egg) or $\frac{1}{1500}$, which means that a drop of test inoculum from a recently inoculated embryo would contain a maximum of 50 LD₅₀ infectious units. These calculations take no account of the probable fixation of the virus on the tissue. It follows that although in the few hours after inoculation of the virus into the allantoic sac we did not recover the "expected" amount (see Table V), we cannot conclude that there was a latent period before its proliferation. Any virus formed may have been fixed in the tissues.

Effect of Age of Embryo

In the preceding paper, it was noted that there was relatively little effect of age on the susceptibility to the virus. This, however, would not mean that the same total amount of virus was obtainable from allantoic fluid of eggs of different ages. The point was tested by the inoculation of 10-, 12-, and 14-day-old embryos into the allantoic sac (Table VI). The fluid was harvested from two to five embryos of each age, and titrations of red cell agglutination indi-

TABLE IV
Rate of Increase of Virus in Allantoic Fluids from Various Amounts of Inoculum

Time	Egg No.	Undiluted allantoic fluid										1/100 dilution										1/10,000 dilution										Embryo infectivity of pool	
		Red cell agglutination of harvested allantoic fluid										Red cell agglutination of harvested allantoic fluid										Red cell agglutination of harvested allantoic fluid											
		1/10	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	Egg No.	1/10	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	Egg No.	1/10	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	Egg No.		
6	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	10 ^{-4.5}	
	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2		
	3	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3		
17	1	0	0	+	++	++	+	0	0	0	1	++	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	10 ^{-4.5}	
	2	0	+	++	++	++	0	0	0	0	2	++	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2		
	3	0	++	++	++	++	0	0	0	0	3	+	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	3		
24	1	0	0	0	++	++	++	+	0	0	1	0	++	++	++	+	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	10 ^{-3.4}	
	2	0	0	0	++	++	++	++	0	0	2	0	++	++	++	++	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2		
	3	0	0	0	++	++	++	++	0	0	3	0	++	++	++	++	++	0	0	0	3	0	0	0	0	0	0	0	0	0	3		
32	1	0	0	0	++	++	++	++	0	0	1	+	++	++	++	++	0	0	0	0	1	+	0	0	0	0	0	0	0	0	1	10 ^{-4.5}	
	2	0	0	0	++	++	++	++	+	0	2	0	++	++	++	++	+	0	0	0	2	++	+	0	0	0	0	0	0	0	2		
	3	0	0	0	++	++	++	++	++	0	3	0	++	++	++	++	++	0	0	0	3	++	++	++	++	++	++	++	++	++	3		
	4	0	0	++	++	++	++	++	++	0	4	0	++	++	++	++	++	++	++	++	4	0	++	++	++	++	++	++	++	++	++		4
43	1	0	+	+	++	++	++	++	++	0	1	++	++	++	++	++	++	0	0	0	1	++	++	++	++	++	++	++	++	++	++	1	10 ^{-4.5}
	2	+	++	++	++	++	++	++	++	0	2	0	++	++	++	++	++	+	0	0	2	++	++	++	++	++	++	++	++	++	++	2	
	3	++	++	++	++	++	++	++	++	0	3	0	++	++	++	++	++	++	++	++	3	0	++	++	++	++	++	++	++	++	++	3	
	4	++	++	++	++	++	++	++	++	0	4	++	++	++	++	++	++	++	++	++	4	++	++	++	++	++	++	++	++	++	++	4	
53	1	0	+	+	++	++	++	++	++	0	1	++	++	++	++	++	++	++	++	++	1	0	0	+	++	++	++	++	++	++	++	1	10 ^{-4.5}
	2	+	++	++	++	++	++	++	++	0	2	++	++	++	++	++	++	++	++	++	2	0	0	++	++	++	++	++	++	++	++	2	
	3	++	++	++	++	++	++	++	++	0	3	++	++	++	++	++	++	++	++	++	3	++	++	++	++	++	++	++	++	++	++	3	
64	1	0	+	+	++	++	++	++	++	0	1	++	++	++	++	++	++	++	++	++	1	0	0	0	+	++	++	++	++	++	++	1	10 ^{-4.5}
	2	+	++	++	++	++	++	++	++	0	2	++	++	++	++	++	++	++	++	++	2	0	0	0	++	++	++	++	++	++	++	2	
	3	++	++	++	++	++	++	++	++	0	3	++	++	++	++	++	++	++	++	++	3	++	++	++	++	++	++	++	++	++	++	3	

TABLE V

Growth of Virus after Inoculation of a 10^{-4} Dilution of Allantoic Fluid at 35° C.

Time	Titer
<i>hrs.</i>	
1½	$10^{+0.2}$
3½	$10^{>0}$
7	$10^{-1.7}$
18	$10^{-4.0}$

TABLE VI

Effect of Age of Embryo on Titer of Virus Obtained from Allantoic Fluid

10 days		12 days		14 days	
Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
$10^{-9.0}$	1/4000	$10^{-9.0}$	1/2000	$10^{-9.0}$	1/1000

cated that there was a slight decrease in the amount of virus obtainable. This was not great enough to be picked up on infectivity measurements.

SUMMARY

The virus of Newcastle disease of chickens resembles those of the encephalitis group in its ability to spread throughout the developing egg and embryo, but it is similar to influenza virus in the high concentration of it found in the allantoic fluid before death. No effect of the size of the inoculum on the final titer of virus in the allantoic fluid was detected. Good growth occurred at temperatures from 35° to 41°C., apparently more rapid at 40°C. than at 35°C. No appreciable development of virus capable of agglutinating red cells but of low embryo infectivity was found. Although virus multiplication was not immediately perceptible after inoculation, this cannot on present evidence be attributed to a real lag phase.

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STUDIES ON NEWCASTLE DISEASE VIRUS

III. CHARACTERS OF THE VIRUS ITSELF WITH PARTICULAR REFERENCE TO ELECTRON MICROSCOPY

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PLATES 8 AND 9

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Electron micrographs of Newcastle virus suspended in allantoic fluid or water show a number of irregular globular forms (1). Filamentous or tailed forms have not been found in these fluids. However, when the virus is transferred to saline and a mount is made directly of this material, tailed or sperm-like forms are found to predominate (2-4).

The identification of certain particles seen in the electron microscope as virus particles is not easy. Even some 10 years after the original description of the tobacco mosaic virus as a long thin rod (5) there is some discussion as to whether these particles represent individual units (6) or aggregates of virus (7). It is not legitimate to accept any spherical particle obtained from a mixture of materials as virus just because it has a size which agrees with previous calculations and because it is frequently found in these partially purified preparations. All available methods for associating or dissociating the two must be applied.

We first would like to outline certain evidence which we believe associates the filamentous or sperm-like bodies seen in saline preparations with the individual virus particles. We will then take up the evidence that the tailed forms are derived from the globular forms in the allantoic fluid.

The Evidence for an Association of Virus Activity with the Filamentous Units

The evidence indicative of an association of filamentous units will be considered under several heads:—

1. *Lack of Similar Forms in Other Infections of the Embryo.*—Several other virus infections have been studied under the electron microscope. The most fully reported is the influenza group (8, 9), in which certain filamentous forms have been described (10). However, these forms are in the minority in the preparations that have been published and have been pictured as long solid threads, never as sperm-like forms of variable thickness. They have not definitely been identified as virus particles. The virus of cat pneumonia has recently been studied in preparations from the allantoic fluid of chick embryos and no filamentous forms are described (11). Eastern and Western encephalomyelitis viruses are purified in higher centrifugal fields but infected chick embryos are used as starting material. No filamentous forms have been de-

scribed (12). We have studied partially purified preparations of swine influenza, mumps, and Eastern equine encephalomyelitis without seeing any forms which resemble the usual picture of Newcastle virus when resuspended in saline. This negative evidence makes it unlikely that the peculiarities observed are ordinary artifacts.

2. *Period at Which Filamentous Forms Are First Obtained.*—Filamentous forms have been obtained from allantoic fluid as early as 24 hours after inoculation. This corresponds to the time of first increase in virus titer of the allantoic fluid (13). The forms are obtainable from that time on until death of the embryo some 24 to 48 hours later, during which period the titer is maintained. They have been found in preparations incubated at 35°, 37°, and 41°C. They are thus obtainable at all times when the virus titer is high. Their presence early in the infection indicates that they are not a degenerate form.

3. *The Forms Are Obtained from Four Different Strains.*—The four strains used in the studies here reported have all shown predominantly a tailed or filamentous form when suspended in saline and dried (Figs. 2, 4, 6). None of them has shown this form in allantoic fluid (Figs. 1, 3, 5). The presence of the tailed form in saline preparations has been reported in another strain from the United States (3) and in the classical Doyle strain (4).

4. *The Forms Are Agglutinated by Specific Antisera.*—The infection of a vertebrate host with a pathogenic organism usually results in antibodies against that organism. On previous evidence we can expect that an organism which has been identified as the causative agent of a disease may be agglutinated by convalescent sera from that disease. Failure for this to happen militates against the identification of the material under test as the infectious agent. Agglutination by convalescent sera is however not conclusive evidence for identification of agent, and disease, as witness the rise of the proteus OX titers in various rickettsial fevers, and the increase of anti-beef heart particle titer in syphilis. An increase in the agglutinating titer against vaccinia particles in rabbits infected with vaccinia (14) and of fowl pox bodies of chickens infected with fowl pox (15) has been demonstrated. Such tests have not hitherto been applied to electron microscope studies.

In the course of the present work, agglutination of Newcastle virus particles was first demonstrated in a $1/6$ dilution of antiserum against the classical strain of the disease.¹ This was controlled by tests with serum from a rooster hyperimmunized with chick embryo preparations of equine encephalomyelitis.

A drop of fluid from each mixture was mounted on an electron microscope screen 15 minutes, 2 hours, and 5 hours after the test was started. Slight beginning clumping at 15 minutes, which became definite in 2 hours, was noted with the microscope in the Newcastle immune sera. The control sera failed to agglutinate even in 5 hours. That the agglutination was not due to a reaction with some normal tissue component of the virus is made likely by the fact that the

¹ I am indebted to Dr. C. A. Brandley for furnishing these sera.

anti-Newcastle sera in the same test failed to agglutinate partially purified influenza virus from chick embryos.

The agglutination test was repeated in another way.

Of four chickens which had been used in the terminal dilution of a titration, two had developed good red cell agglutination inhibition titers (presence of infection) and two failed to develop antibodies (no infection). To $\frac{1}{2}$ cc. of serial two fold dilutions of serum, 0.1 of a cc. of virus concentrate was added. The concentrate had been prepared by centrifuging the virus from the allantoic fluid and resuspending in saline equal to 0.1 the original volume of allantoic fluid. The combination was put in the refrigerator overnight.

As shown in Table I the sera from the infected chickens agglutinated macroscopically the virus suspension in dilutions of $\frac{1}{16}$ and $\frac{1}{32}$. The control sera failed to agglutinate. Electron microscope pictures prepared 1 hour after the

TABLE I
Macroscopic Agglutination of Newcastle Virus Suspension

Sera and virus concentrate in saline were put in refrigerator overnight.

Chicken sera used	No.	Dilution of sera					
		1/4	1/8	1/16	1/32	1/64	1/128
Known positive sera	1	+++	+++	+++	++	0	0
	2	+++	+++	++	0	0	0
Known negative sera	1	0	0	0	0	0	0
	2	0	0	0	0	0	0

addition of the sera showed, in the $\frac{1}{4}$ and $\frac{1}{8}$ dilutions, masses of clumped virus with no discrete particles left. The $\frac{1}{16}$ dilution of both positive sera showed some free particles. No microscopic agglutination (electron microscope) was seen in any dilution of the controls.

5. *Association of Infectivity and Red Cell-Agglutinating Activity with Particles of about 100 m μ .*—It was early demonstrated by filtration experiments that Newcastle virus has a diameter between 80 and 120 m μ (16). It was to be expected then that centrifugation at a force sufficient to concentrate influenza virus would throw down Newcastle virus. This was found to be so (2). Indeed it has been demonstrated (3) that a force of 15,000 to 20,000 g for 15 to 20 minutes is sufficient to sediment the virus particles (as determined by red cell agglutination). This accords with the size of the particles seen by electron microscopy (17).

For the sake of completeness, we present data on three other strains of the virus, demonstrating the ease of sedimentation and concentration. These tests were carried out in a higher gravitational field than necessary and therefore do not indicate as clearly as does the work of Cunha *et al.* (3) that the size of the infectious particle is about 100 m μ (Table II).

Although the red cell-agglutinating activity of the influenza-mumps-Newcastle group of viruses is much more stable than the infectivity of the virus for the embryo, it has not been possible as yet to separate the red cell agglutinating factor from the 100 m μ particle. In an attempt to do this we studied the effect of freezing and thawing on the sedimentability of the virus (measuring both red cell and embryo infectivity). It was consistently found that freezing and thawing of a preparation of infected allantoic fluid caused a much larger amount of virus to remain suspended in the supernatant fluid after centrifuging at 24,000 R.P.M. for 40 minutes (30,000 g minimal) (Table III). Both the infectivity and red cell-agglutinating titers of the top layer of supernatant were about ten times greater when the material had been frozen and thawed beforehand. There are several possible explanations of this phenomenon. (It is, of course, understood

TABLE II

Sedimentation of Several Strains of Newcastle Virus

Centrifugation at 24,000 R.P.M. (30,000 g⁺) for 30 minutes.

Strain	Original fluid		First supernatant		Pellet resuspended after second centrifugation	
	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
Cg179		1/40		0	10 ^{-5.5}	1/80
	10 ^{-7.8}		10 ^{<5.5}		10 ^{-5.1}	
	10 ^{-5.4}	1/10	10 ^{-5.7}	0	10 ^{-5.3}	1/80
Np	10 ^{-5.5}	1/1600		0	10 ^{-5.5}	1/800
W		1/400		1/10	10 ^{-5.0}	1/400

that the flocculi and clumps of virus and protein due to freezing and thawing have in each instance been broken up by repeated pipetting).

It is conceivable that the inherent viscosity of the suspending medium has been increased by freezing and thawing. This might change the character of the convection currents in the tube and might also directly affect the sedimentation rate of the virus. Such a state of affairs is unlikely because after a second centrifugation at 24,000 R.P.M. in which the virus was in saline the supernatant from the preparation—which had originally been frozen and thawed,—still contained a higher hemagglutinating titer than the control supernatant. Another explanation is that the virus has in some way been altered so that it does not sediment as well. This could be consequent either on the formation of smaller particles (a breaking up of perhaps 10 per cent of the virus particles) or on the change from the compact spherical to the larger filamentous shape, which seems to occur when the virus is transferred from allantoic fluid to saline solution (1).

Such a change might also occur in unfrozen preparations after standing. However, an apparent change in size after freezing and thawing may not indicate the breaking up of a proportion of the individual particles, but instead the separation of agglomerate masses. This would explain the frequent increase in total titer of virus obtained when purifying this virus (2) and that of influenza (18).

6. *Infection Is Produced by Very Few Particles.*—If infection of the host is produced by an inoculum containing a very few particles, perhaps only one, it is likely that a single particle is the infecting agent (19). If, however, the mini-

TABLE III
Effect of Freezing and Thawing on Sedimentability of Newcastle Virus

Experiment	Original		Supernatant		Pellet resuspended in same volume saline after 1 centrifugation 40 min.	
	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
1 Unfrozen	$10^{-8.8*}$	1/3200	$10^{-8.8}$	1/24	$10^{-8.8}$	1/3200
Frozen		1/6400	$10^{-8.9}$	1/384	$10^{-8.8}$	1/3200
2 Unfrozen				1/24	$10^{-8.8}$	1/3200
Frozen				1/384	$10^{-8.8}$	1/3200
3 Unfrozen			$10^{-8.8}$	1/32	$10^{-8.8}$	1/800
Frozen			$10^{-7.2}$	1/512	$10^{-8.8}$	1/800
4 Unfrozen	$10^{-9.0}$	1/3200	$10^{-4.5}$	1/16		1/3200
Frozen slowly	$10^{-8.4}$	1/3200	$10^{-8.4}$	1/128		1/3200
Frozen quickly	$10^{-8.8}$	1/3200	$10^{-7.5+}$	1/1024		1/1600

* This material titered to $10^{-8.8}$ after three freezing and thawing treatments.

imum infecting dose contains thousands or millions of particles of the calculated size, there is more room for error in identifying the particle as the infecting agent.

The evidence that a very few particles of Newcastle virus produce infection and death of the embryo is threefold. (a) Calculation of the number of particles in a suspension, based on the size of the particle in the electron microscope, the light scattering, and the nitrogen content of the suspension, shows that there are only a few more particles than there are infectious units. The difference between the two measurements is well within the limits of experimental error. (b) Equally high titers may be obtained in two different hosts, and (c) end-point titration curves agree with the single particle infection theory. None of these lines of evidence is in itself conclusive but taken together they make it

likely that the particles seen under the electron microscope are the virus. To deal with these points individually:

(a) Electron microscope measurements of the size of head of the large fairly uniform particles in the purified preparation average $83 \text{ m}\mu \times 146 \text{ m}\mu$. Light-scattering measurements (20) combined with nitrogen determination on washed virus lead to an estimate of 115 to 120 $\text{m}\mu$ in size,² and a molecular weight of about 500 million. In a suspension containing 1 gm. of protein, there would then be roughly 1.2×10^{16} particles. It has, however, been shown that 1 gm. of virus (assuming $N = 1/10$ of total mass) will titer to $10^{-14.6}$ (50 per cent end-point). There must then be about five particles present at the 50 per cent end-point.

(b) Parker (19) has pointed out that when a given virus suspension attains the same high titer and reaches the same end-point in three different hosts (vaccinia in rabbit, mouse, and chick embryo), it is probable that infection is produced by one particle. The strain Cg179 which is highly virulent for chickens was therefore simultaneously titrated in 10-day chick embryos and young chickens (Table IV). There

TABLE IV
Titer of Cg179 on Chick Embryos and in Chickens

Preparation No.	10 days embryos	2 wks. chicks	2 mos. chickens
1	$10^{-8.8}$	$10^{-8.6}$	
2	$10^{-8.1}$	$10^{-8.2}$	
3	$10^{-7.2}$		$10^{-6.8}$
4	$10^{-7.5}$		$10^{-6.7}$

was no detectable difference in result on the 10-day chorioallantoic membrane and on the 2 weeks-old-chicken. There was a slight decrease (of questionable statistical significance) when 2 months-old-chickens were inoculated. We have then two hosts of roughly equal susceptibility to high dilutions of virus.

(c) The theory that infection is produced by one infectious unit has been extensively discussed (21) and the expected curve for such a case outlined. Our results (22, Chart 1) agree with this predicted curve. This type of curve at best merely indicates that when infection has been produced in the embryo it may have been initiated by one particle. It does not take into account the virus particles which have been lost. These amount to thousands in the case of tobacco mosaic virus.

Points (b) and (c) are brought into the discussion not because of any belief that they prove the identity of the demonstrated particle and infectious unit, but because they agree with the evidence in (a) that infection is produced by a very few particles.

Apparent Change in Shape of Infectious Particle

If we accept the sperm-like or filamentous forms of Newcastle virus, when prepared from saline suspensions, as representing actual virus particles, how

² Actual measurements and calculations of size were carried out by Dr. Roger Herriott.

may we then explain their absence in the original allantoic fluid? Saline suspensions in which the virus is not concentrated show many typical tailed forms, whereas none are seen in allantoic fluid having this same titer of virus. We believe that the virus particle has another form in the allantoic fluid and that under the influence of saline it is changed into the forms just mentioned. The evidence in favor of this is as follows:—

1. Filamentous forms are absent from the allantoic fluid.
2. Filamentous forms can be produced by simple dialysis against saline. This is accomplished without loss of activity.
3. Suspensions maintained in different pH solutions give different forms.
4. The form of virus suspended in a phosphate buffer at pH 8 differs from that suspended in a borate buffer at the same pH.
5. Spherical forms can be obtained from allantoic fluid by resuspending in water. These will show filamentous types under the electron microscope if increasing concentrations of saline are added.
6. Partial inactivation of spherical forms in water by heat, formaldehyde, or mustard gas prevents the development of the filamentous forms.

It may be worth while to detail the experimental evidence on each of these points.

1. *Absence of Filamentous or Tailed Forms from Allantoic Fluid.*—Numerous unsuccessful attempts were made to see these forms in the unconcentrated allantoic fluid.

To preclude the possibility that the mechanical treatment of centrifugation and resuspension might cause the change in shape, normal allantoic fluid was substituted for saline during both cycles of centrifugation of one strain. No tailed forms were found after a careful search of many fields. The same concentration of virus in saline had produced great numbers of tailed forms in each field. This contrast between allantoic-fluid virus and saline-washed virus held true of all four strains.

2. *Production of Filamentous Forms by Dialysis against Saline.*—Filamentous forms may be observed to develop within the original allantoic fluid when it is dialyzed against saline solutions. These forms do not have the clear cut morphology of the typical tailed virus which has been centrifuged and transferred to saline, but do show a definite change in morphology. In the first experiment, infected allantoic fluid was put in a cellophane sac under slight pressure and fluid withdrawn at intervals of several hours. Filamentous forms were found during the first 12 hours. In the two subsequent experiments the virus was dialyzed against saline and water. The infectiousness of the preparations was determined and electron microscope screens prepared at intervals. The data are summarized in Tables V and VI.

We have concluded that a definite change in morphology may be brought out by dialysis against saline with little loss of infectiousness, but since other methods of investigating this morphological variation proved capable of bringing out sharper differences, we did not pursue this aspect further.

3. *Effect of pH.*—When virus is transferred from allantoic fluid to a saline solution by centrifugation and resuspension, it is transferred from a medium of roughly pH 8 to one of roughly pH 7—especially if the saline is buffered by phosphate. It was im-

portant then to determine the effect of various hydrogen-ion concentrations on the morphology and activity of the virus.

Infected allantoic fluid was centrifuged in the usual manner and the virus resuspended in one-tenth of the original volume of normal saline. This then was brought up to the original volume of normal saline. This then was brought up to the original volume in the particular buffered solution desired by a one to ten dilution. pH recordings are actual determinations with Coleman pH electrodes dipped in the virus solution.

TABLE V
Dialysis against 0.15 M Saline
No pressure

Effect on	Original	Time, hrs.					
		2	10	50	70	94	216
Morphology.....	Spherical	Spherical	Fuzzy		Stringy		
Titer.....	$10^{-8.5}$		$10^{-8.8}$	$10^{-8.5}$	$10^{-8.4}$	$10^{-8.5}$	$10^{-8.4}$

TABLE VI
Dialysis through Cellophane Sac
Original titer $10^{-8.5}$. No pressure

Dialysed against	Effect on	Time, hrs.			
		6	24	72	216
Normal 'sa- line	Morphology	Fuzzy and stringy	Indistinct tailed forms		Filamentous
	Titer			$10^{-8.5}$	$10^{-8.2}$
Buffered sa- line	Morphology	Stringy			
	Titer		$10^{-8.8}$		
Water	Morphology	Large irregu- lar dots		Large irregu- lar dots	
	Titer			$10^{-8.4}$	

Since the changes were greater on the alkaline side of pH 7 (Table VII), the second experiment was limited to this. The data obtained are presented in Table VIII.

Several things seemed clear from these experiments on the effect of pH. First, filamentous forms were not seen in moderately alkaline solutions even though activity was well preserved. Secondly, the tailed and filamentous forms were not preserved indefinitely at any of the pH concentrations tested. Finally it also seemed clear that it was not the change from pH 8 to pH 7 in itself which caused the appearance of the filamentous forms but rather some change in salt concentration.

4. *The Effect of Phosphate Buffer.*—Further evidence of the secondary importance of hydrogen ion concentration *per se* was obtained by studying morphology and activity at pH 7.0, 7.5, and 8.0 all in phosphate buffers (previous studies on the effect of pH had used a borate buffer for pH 8). Filamentous and tailed forms predominated at all three pH's and in two different strains of virus (B and Cg179).

TABLE VII
Effect of Different Hydrogen Ion Concentrations on the Morphology and Activity of Washed Virus

pH	Effect on		Time			
			2 hrs.	1 day	12 days	19 days
3	Activity	Embryo titer Red cell titer	$10^{-8.0}$	$10^{-8.5+}$	$10^{-4.8}$ 1/100	
	Morphology			No filamentous forms seen		
5	Activity	Embryo titer Red cell titer		$10^{-7.5}$	$10^{-8.5}$ 1/800	$10^{-5.3}$
	Morphology				Few filamentous	Spherical, many with tails
7	Activity	Embryo titer Red cell titer	$10^{-6.6}$		$10^{-9.0}$ 1/800	
	Morphology		Typical filamentous	Tailed and filamentous		Spherical
8.0	Activity	Embryo titer Red cell titer		$10^{-8.4}$	$10^{-8.5}$ 1/400	
	Morphology				Spherical	No filamentous
8.8	Activity	Embryo titer Red cell titer		$10^{-8.5+}$	$10^{-8.5+}$ 1/800	
	Morphology			Filamentous		No filamentous. Outlines vague
9.2	Activity	Embryo titer Red cell titer	$10^{-5.1}$	$10^{-7.5+}$	$10^{-7.5+}$ 1/1600	$10^{-5.6}$
	Morphology		Hazy, and crystals	No filamentous	Sharply defined hard dots	Sharply defined hard dots

5. *Effect of Molarity of Salt Solution.*—Infected allantoic fluid contains a great deal of extraneous material besides the virus particles. This makes it difficult to differentiate individual particles and to identify each virus particle. For this reason we felt that the evidence for a change in form would remain obscured until it was possible to obtain spherical particles free from the original allantoic fluid. To this end we undertook to study the morphology and infectivity of Newcastle virus in distilled water. Surprisingly the virus can be centrifuged and resuspended in water without loss of

infectivity. It has a spherical shape which is readily converted into a tailed or filamentous form by the addition of 0.07 to 0.15 molar sodium chloride.

The experiments summarized in Table IX were performed by first resuspending the pellet obtained from ultracentrifugation in water. This washing in water was repeated, and the second centrifugate was resuspended in one-half to one-sixth of the original fluid volume. This water suspension was then brought back to approximately the original volume in the particular strength of saline desired. Suspensions were stored at 4°C.

TABLE VIII
Effect of pH on the Morphology and Activity of Newcastle Virus

pH	Effect on	2 hrs.	2 days	4 days	12 days	16 days
6.8	Activity		$10^{-8.3}$		$10^{-7.5}$	
	Morphology	Filamentous	Filamentous	Filamentous	Few filamentous	Rare filamentous
8.0	Activity				$10^{-7.5}$	
	Morphology		Rings	Small hard dots		Irregular mixture
8.8	Activity		$10^{-8.3}$		$10^{-8.0}$	
	Morphology	Filamentous and tailed	Spherical	Large with vague outline		Large round spheres
9.2	Activity		$10^{-8.3}$		$10^{-7.5}$	
	Morphology		Small dots	Irregular in outline		
9.3	Activity		$10^{-8.4}$			$10^{-7.5}$
	Morphology		Small dots			Dense round small spheres

Figs. 7 to 12 show the gradual transition from the spherical to the filamentous, with no measurable change in infectiousness, as shown in Table IX.

The ability of saline to bring about these changes in form as seen on electron microscopy immediately raises the question of effect through various osmotic pressures. It must be remembered, however, that as the drop of saline containing the virus particles dried at room temperature on the collodion film of the electron microscope screen there was an increase in salt concentration which makes it impossible to state exactly what the osmotic pressure was during the drying. However, it has been found that virus suspended in 0.15 M sucrose is just as active as the original allantoic fluid and has a spherical form on electron microscopy. It may be worth pointing out that the effect of the salt solution seems to be quick for the filamentous forms were present within 10 minutes of adding the saline plus the time taken in drying (5 to 10 minutes).

TABLE IX
Effect of Molarity of Saline on Morphology and Activity of Virus

Experiment No.	Time	Effect on	H ₂ O	Molarity of saline					
				0.05	0.1	0.15	0.2	0.25	0.3
1	1 day	Embryo infectivity	10 ^{-3.3}	10 ^{-3.6}		10 ^{-3.0}			10 ^{-7.2}
		Red cell agglutination Morphology	12,800 Spherical	12,800 Spherical	6,400 Spherical	6,400 Tailed	6,400 Heavy filaments	1,600 Heavy filaments	1,690 Clumps*
	4 days	Embryo infectivity Morphology	10 ^{-3.7} † Few asymmetrical. None filamentous	10 ^{-3.7}	Mostly spherical	10 ^{-3.2} Thick filaments		Large vague forms, few tails	10 ^{-4.0} Clumps*
2	½ hr.	Embryo infectivity Morphology	10 ^{-3.3} Spherical	(0.07 M) Tailed		Filamentous and tailed	Filamentous and tailed	Filamentous and tailed	
	5 days	Embryo infectivity Morphology	10 ^{-3.3} Spherical						
	3 10 min.	Morphology	Spherical‡	(0.07 M) Tailed forms beginning		Filamentous forms thick	Filamentous	Filamentous and tailed	

* Clumps visible grossly.

† Determined after 8 days in water.

‡ Gold-shadowing revealed a few short thick tails projecting from spheres.

This agrees with the immediate appearance of filamentous forms on transfer into saline during the original centrifuge studies described in the first part of this paper.

6. *Effect of Partial Inactivation of Spherical Forms on the Ability of the Virus to Change Shape.*—The question which was constantly in the background was, —Is this change a real one, or is it due to some peculiar distortion of the virus on drying? It was thought that if it were possible to prevent the change in shape brought about by saline by some previous treatment of the virus in the water suspension, this would make it less likely that we were dealing with an artifact. To this end, 0.02 per cent formaldehyde was added to a water suspension of virus and this suspension kept at 4°C. for 4 days. Sufficient NaCl solution was then added to produce a 0.15 M solution and immediately thereafter and 5 minutes afterwards screens were prepared for electron microscopy.

TABLE X

Effect of 0.02 Per Cent Formaldehyde on the Change of Shape Brought about by Saline

Time	Water suspension		0.02 per cent formaldehyde in water	
	Activity	Morphology when placed in 0.15 M saline	Activity	Morphology when placed in 0.15 M saline
hrs.				
0	10 ^{-8.7}	Filamentous		
1			10 ^{-8.3}	Filamentous and tailed
4			10 ^{-7.5+}	Filamentous and tailed
27			10 ^{-7.5+}	Spherical and filamentous
72			10 ^{-8.5+}	Most spherical
120'		Filamentous and tailed	10 ^{-8.5+}	Most spherical; few filamentous
288	10 ^{-8.7}	Few filamentous	10 ^{-8.5}	

The typical spherical forms of the water suspension were preserved by the previous treatment with formaldehyde despite the presence of the sodium chloride. Only a rare tailed virus particle was seen on thorough search of the screen. The failure to find forms other than spherical in a preparation of virus which had been treated with formaldehyde before centrifugation was reported by Taylor (23) at the Electron Microscope Society Meeting in 1946. On the other hand, a control suspension of virus to which saline was added before placing in the refrigerator, and to which 0.02 per cent formaldehyde was added after 4 days at 4°C., showed a profusion of tailed forms. This would indicate that it is not the mere presence of formaldehyde which prevents visualization of tailed forms, but that it is necessary for the formaldehyde to act over a period of time.

This idea was tested by comparing the ability of the spherical forms to change into tailed and filamentous forms with and without 0.02 per cent formadehyde over a period of time. The results are presented in Table X. The electron microscope screens were prepared within a few minutes after adding the saline.

It is apparent that roughly the same results were obtained. 0.02 per cent

formaldehyde did not inhibit the change in shape until after some days during which time it had a chance to begin to inactivate the virus. However, conversion seemed to be inhibited long before inactivation had proceeded very far. It is not surprising then that the control tube in water alone produced but few filamentous forms after 12 days in the refrigerator, at which time roughly 99 per cent of the virus was apparently inactivated.

One other method of inactivation was studied fairly carefully for its ability to prevent the conversion into filamentous forms. Water suspensions were heated in a water bath at 50°C. for various periods of time. Portions were then brought to 0.15 M NaCl concentration and electron microscope screens were prepared. As can be seen in Table XI, long before inactivation was complete

TABLE XI
Effect of Heating (50°C.) on Change to Filamentous and on Infectivity of Virus

Experiment No.	Time	Morphology when transferred to saline	Titer
	min.		
1	0	Filamentous	10 ^{-3.7}
	10	No tailed forms seen; few asymmetrical	10 ^{-6.0}
	20	None filamentous or tailed	
	30	Beginning aggregation; outlines indistinct	
	60		10 ^{-1.0+}
2	0	Excellent filamentous forms	
	1	Most forms tailed or filamentous	
	5	Few scattered filamentous forms, some with long thin tails	
	10	Hard dense forms. Outlines irregular but essentially spherical. Few clumps	
	15	Dense hard forms, most spherical. No filamentous forms	

as far as infectivity was concerned, conversion was prevented. It is well to remember here, as in the experiments on the effect of formaldehyde, that the loss of only 3 logs in a titration means a 99.9 per cent inactivation. This amount of inhibition of conversion as seen in the electron microscope would be called complete. In a second experiment the amount of heating necessary for this inhibition was more accurately determined, but no activity determinations were made. Again it was found that 10 minutes at 50°C. prevented the conversion.

Two other methods of inactivation were studied in passing. Irradiation of water suspensions with ultraviolet light for 20 minutes in one experiment and for 60 minutes in another prevented conversion. Five to 10 minutes of irradiation under the same conditions had been found to render the virus non-infectious for the embryo. Treatment with mustard gas at a concentration of 2×10^{-4} M (24) also made the virus incapable of producing the filamentous form. In general reactions which inactivated the virus were capable of preventing the conversion, perhaps to the same degree.

DISCUSSION

Two separate ideas have been presented in this paper. The evidence for the first, that certain filamentous and tailed forms represent virus particles, is fairly good. It is based on their characteristic shape, on their size and number, and on immunological evidence.

The second idea, that there is a genuine change in shape from a spherical to a filamentous one, is a more difficult one. While repetition readily establishes the reproducibility of the phenomenon, it does not discourage the idea of a reproducible artifact. This problem may be debated at length and involves the basic problem of the applicability of electron microscope findings in the biological field. Does the form or shape of the virus of Newcastle disease *in solution* depend upon the presence or absence of a certain concentration of salt? It might be thought that the best way to answer this would be by applying certain known physical methods to a study of the virus suspensions. Light-scattering measurements were made by Dr. R. M. Herriott on the virus in allantoic fluid and in saline. No change was found. Then the scattering of light was determined in purified suspensions before and after adding salt. No change was found. But we were unable to get any information which would indicate how much change in shape without change in size might occur without a change in the light scattering.

The asymmetry of particles such as the tobacco mosaic virus is easily established as occurring in solution by birefringence on streaming. In a capillary tube supported between two crossed prisms it was easy to demonstrate the marked streaming birefringence of a 1 per cent suspension of tobacco mosaic virus. This same set up failed to elicit birefringence on streaming in a Newcastle virus suspension in saline which had fifteen times the original concentration in the allantoic fluid and which was a milky white in color. But we cannot conclude from this that the filamentous forms do not exist in suspension. The electron microscope does not indicate that they in any way approach the sharp rigid rod-like shape of tobacco mosaic.

A third method of studying the shape of macromolecules in solution is to study their viscosity. According to Einstein's formula a change in shape should be accompanied by a definite change in viscosity. But in order to get determinable differences of viscosity between the solvent and the solvent plus virus, Dr. Herriott found it necessary to employ concentrations of virus ten times as great as those used in preparing the electron microscope screens. This increased concentration seems to be accompanied by such agglomeration of the individual particles that again the failure to determine a difference between the viscosity of the virus in water and in saline is not significant.

Other physical methods will have to be employed before we can make a final decision. In the meantime the reproducibility and the marked extent of the change, plus the effect of partial inactivation of the virus favor the reality of the phenomenon.

If the change in shape is found to occur in solution it may be related to an enzyme like the ribonuclease which was found to be active in saline and inactive in water solutions.

In passing it is well to emphasize that we have studied and discussed a relatively simple question. Does the virus change from a roughly spherical particle to a roughly filamentous particle under the influence of saline? This excludes the problem of pleomorphism. Our observations would rather indicate that the Newcastle virus is pleomorphic much like the pleuropneumonia bodies. The range of such variation in shape has not been studied.

SUMMARY

1. It is likely that certain tailed and filamentous particles seen on electron microscope examination of partially purified saline suspensions of Newcastle virus are the individual virus particles because:

(a) They have a highly characteristic shape not seen in other virus preparations.

(b) They are present whenever the virus is present in high concentration.

(c) Their size agrees with the size of the virus as calculated from light scattering and centrifuge data.

(d) They are agglutinated by specific antisera.

(e) Infection may be produced in the embryo by relatively few of these particles.

2. It is possible that these filamentous forms have been derived from spherical forms without loss of activity because:

(a) Such filamentous forms are not found in the original allantoic fluid when this contains a comparable amount of virus.

(b) Filamentous forms appeared in the original allantoic fluid when it was dialyzed against saline solution.

(c) Filamentous forms were produced at certain hydrogen ion concentrations but not at others, in solutions maintaining the same infectivity for the embryo.

(d) Spherical forms were obtained by suspending the partially purified virus in water instead of saline. In this the virus remained moderately stable.

(e) These round forms could apparently be converted into tailed and filamentous forms by the addition of saline, again without loss of activity.

(f) This "conversion" could be inhibited by partial inactivation of the water suspension of virus.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Newcastle virus (strain Np) in crude allantoic fluid. $\times 18,000$.

FIG. 2. Same strain in same concentration but partially purified by centrifugation and resuspension in 0.15 M saline. $\times 18,000$.

FIG. 3. Newcastle virus (strain W) in crude allantoic fluid. $\times 18,000$.

FIG. 4. Strain W prepared from 0.15 M saline. Same concentration as Fig. 3. $\times 18,000$.

FIG. 5. Strain Cg179. After centrifugation twice and resuspension in water. $\times 18,000$.

FIG. 6. Same preparation but resuspended in 0.15 M saline. $\times 18,000$.

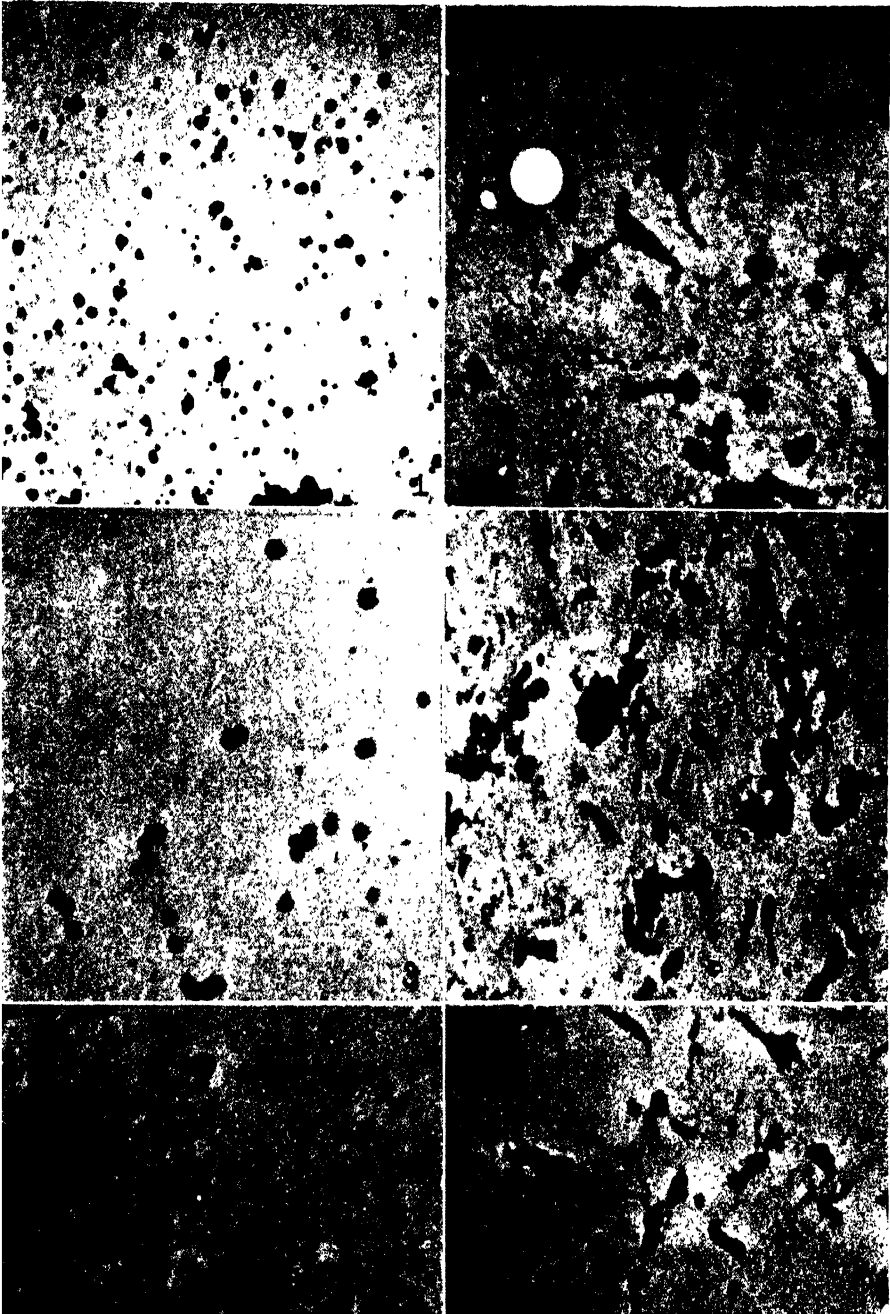


PLATE 9

FIG. 7. Newcastle disease virus (strain B) washed and resuspended in water.
× 18,000.

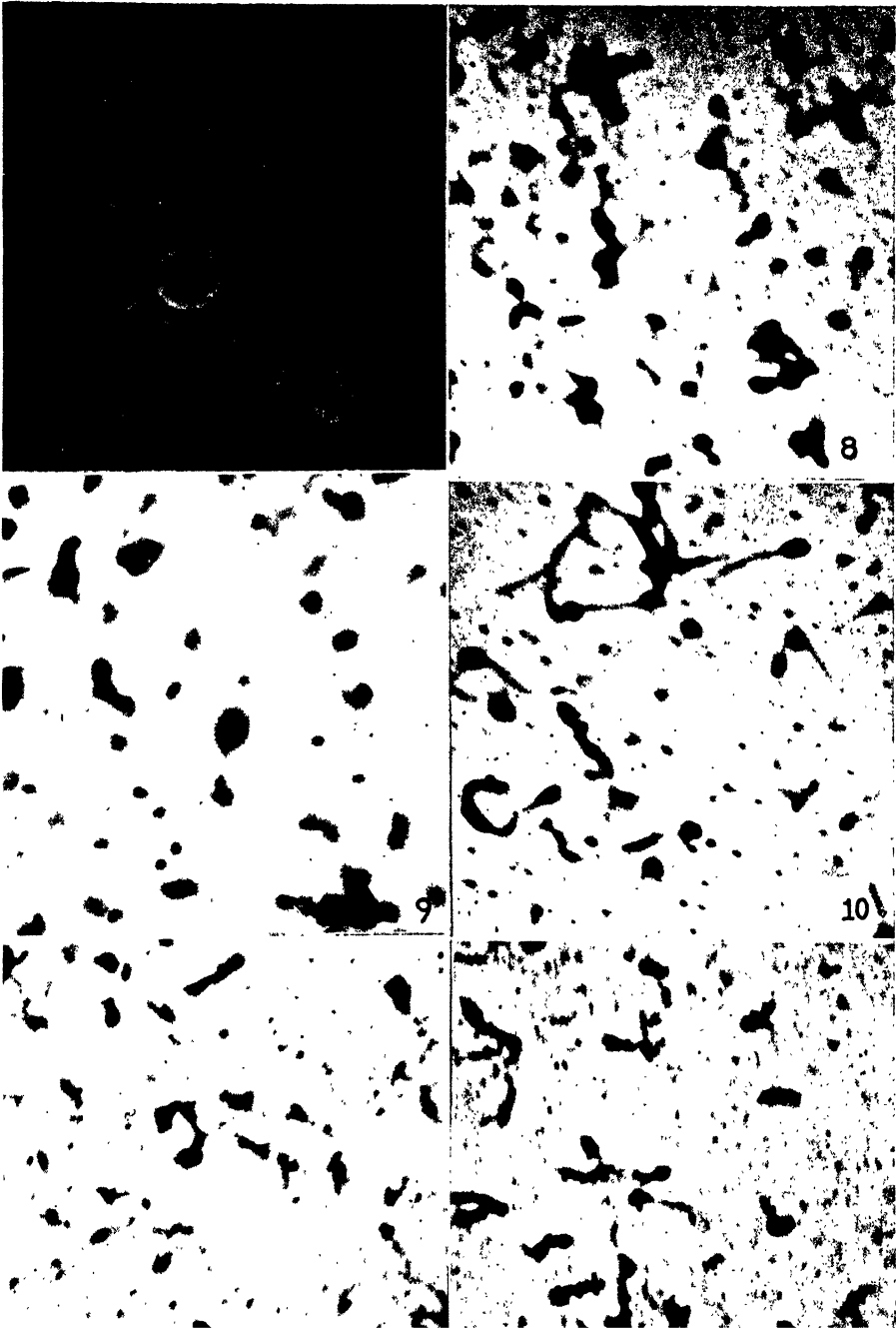
FIG. 8. Same at 0.05 M NaCl. × 18,000.

FIG. 9. Same at 0.10 M NaCl. × 18,000.

FIG. 10. Same at 0.15 M NaCl. × 18,000.

FIG. 11. Same at 0.20 M NaCl. × 18,000.

FIG. 12. Same at 0.25 M NaCl. × 18,000.



THE EFFECTS OF LYSOLECITHIN ON THE GROWTH OF LACTOBACILLUS CASEI IN RELATION TO BIOTIN, PANTOTHENIC ACID, AND FAT-SOLUBLE MATERIALS WITH BIOTIN ACTIVITY

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Williams and Fieger (1946) found that *Lactobacillus casei* could be grown continuously in a medium containing only traces of biotin if oleic acid was present at a suitable concentration. Trager (1947) observed a similar effect with a neutral oil (designated FSF) obtained from hydrolyzed horse plasma. This material, which had some biotin activity for chickens as well as for bacteria, permitted full growth of *L. casei* even in the presence of enough fresh egg white to inactivate completely any traces of biotin which might have been present in the medium. These results have been confirmed and extended (Williams and Fieger, 1947; Axelrod, Hofmann, and Daubert, 1947; Hofmann and Axelrod, 1947; Williams, Broquist, and Snell, 1947).

Lecithin exerts growth-stimulating effects on the lactic acid bacteria (Bauernfeind, Sotier, and Bowff, 1942; Strong and Carpenter, 1942) and can replace oleic acid for organisms requiring this material (Williams, Broquist, and Snell, 1947). It can also replace biotin in the growth of *L. casei* (Trager, unpublished). Since the effects of lecithin may be supposed to be a result of its oleic acid content, it seemed of interest to investigate the effects of lysolecithin, which differs from lecithin in that it does not contain an unsaturated fatty acid.

Materials and Methods

Two samples of lysolecithin, both obtained from the Levene collection of the Rockefeller Institute, have been used. One, no. 1070, contained 3 to 5 per cent amino nitrogen, whereas the other, no. 1072, contained 5 to 10 per cent amino nitrogen. A stock solution of each, containing 2 mg per ml, was prepared in phosphate buffer of pH 7.4 and stored in the refrigerator. Preparation 1070 gave complete hemolysis of washed sheep red blood cells down to a concentration of 1:10,000, whereas preparation 1072 gave complete hemolysis at concentrations down to 1:50,000. The two preparations were, however, identical in their effects on the growth of *L. casei*. Since more of preparation 1070 was available, it was used for most of the experiments.

Stock cultures of *L. casei* were carried by weekly transfer in a medium con-

sisting of 1 per cent yeast extract, 1 per cent glucose, 0.5 per cent peptone, and 1.5 per cent agar. The synthetic media used for the experiments and the method of inoculation were essentially those of Landy and Dicken (1942) slightly modified (Trager, 1947). The experimental cultures were incubated at 37 C for 4 days. Growth was measured by titration with 0.1 N sodium hydroxide.

RESULTS AND DISCUSSION

In a medium containing suboptimal amounts of biotin but an excess of all the other essential growth factors, lysolecithin inhibits the growth of *Lactobacillus casei*. Increasing the concentration of biotin counteracts the inhibitory effect.

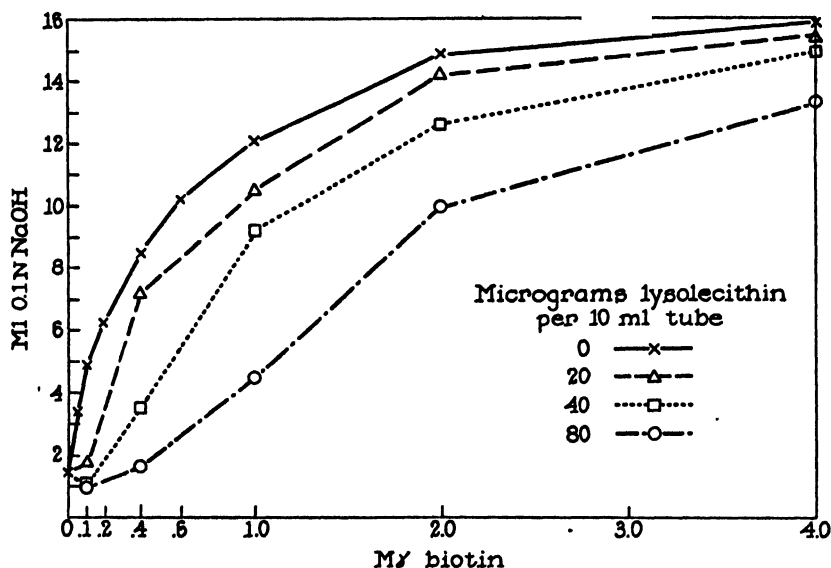


FIG. 1. The inhibitory effect of lysolecithin on the growth of *Lactobacillus casei* in the presence of different concentrations of biotin.

The results of a typical experiment are shown in figure 1. Lysolecithin and biotin, though structurally unrelated, behave toward each other like competitive metabolites, at least so far as the growth of *L. casei* is concerned. A number of instances of competition of this type between structurally dissimilar compounds have been previously reported (Woolley, 1947). It is possible to calculate a molar inhibition index (ratio of moles of inhibitor [lysolecithin] to moles of metabolite [biotin] which will just prevent growth) for the antagonism between lysolecithin and biotin. This index has varied in different experiments between 50,000 and 100,000. For example, in the experiment shown in figure

1, 20 μg of lysolecithin just prevented growth in the presence of 0.1 $\text{m}\mu\text{g}$ of biotin, while 80 μg of lysolecithin were effective against 0.4 $\text{m}\mu\text{g}$ of biotin. In both cases the molar inhibition ratio is 100,000. In another experiment almost complete inhibition of growth was obtained by 16 μg of lysolecithin in the presence of 0.1 $\text{m}\mu\text{g}$ of biotin and by 30 μg in the presence of 0.2 $\text{m}\mu\text{g}$ of biotin, the

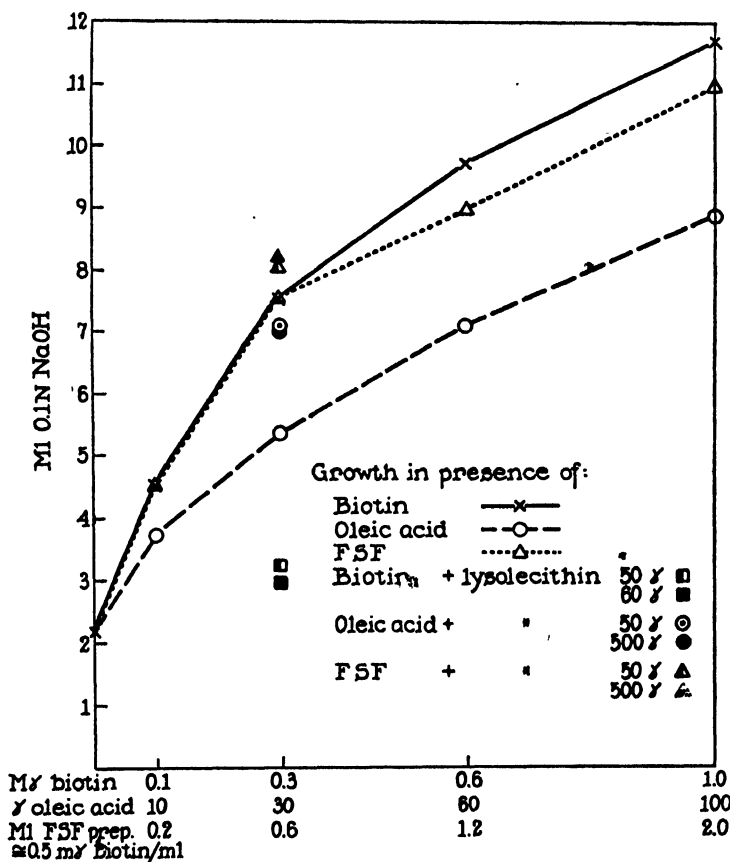


FIG. 2. The growth response of *Lactobacillus casei* to biotin, oleic acid, and FSF, and the effect of lysolecithin in the presence of each of these.

molar inhibition index being 75,000. However, if the biotin of the medium was replaced by various appropriate concentrations of either oleic acid (USP) or FSF from hydrolyzed horse plasma, lysolecithin had a small growth-stimulating effect. This result is well illustrated by the experiment shown in figure 2. Note that whereas 50 or 60 μg of lysolecithin almost completely prevented growth in the presence of 0.3 $\text{m}\mu\text{g}$ of biotin, the same concentration or even ten

times as high a concentration of lysolecithin stimulated growth in the presence of concentrations of FSF or oleic acid of roughly similar biotin activity. This experiment incidentally illustrates the fact that although FSF, like certain esters of oleic acid (Williams and Fieger, 1947), gives a response curve which almost parallels that with biotin, oleic acid gives an appreciably lower curve, probably

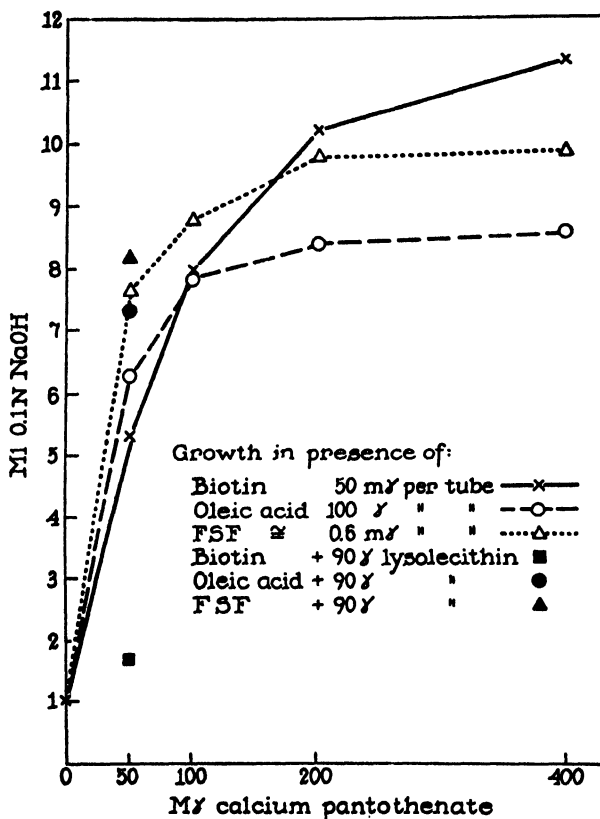


FIG. 3. The growth response of *Lactobacillus casei* to calcium pantothenate in media containing biotin or, in place of it, either oleic acid or FSF, and the effect of lysolecithin in each of these media.

because of its greater toxicity. The stimulatory effect of lysolecithin in the presence of FSF or oleic acid may be the result of a detoxification similar to that described for lecithin and some other materials (Kodicek and Worden, 1945).

Although oleic acid and certain other fatty compounds can fully replace biotin in the nutrition of some of the lactobacilli, they cannot replace riboflavin or pantothenic acid. In the absence of either of these growth factors, or in the

presence of optimal concentrations of both, oleic acid and related materials have no growth-stimulating effect. But in the presence of suboptimal concentrations of either, they have a marked effect (Bauernfeind, Sotier, and Bowff, 1942; Strong and Carpenter, 1942). It was therefore not surprising to find that lysolecithin behaved toward calcium pantothenate, as well as toward biotin, as a competitive inhibitor. This could be clearly shown by using a medium with an excess of biotin and graded concentrations of calcium pantothenate and lysolecithin. In this case the molar inhibition index was only 500 to 1,000. If the biotin of the medium was replaced by either FSF or oleic acid at optimal concentration (an excess could not be used since the higher concentrations of these materials are toxic), the response curve to graded amounts of pantothenate was much steeper. Moreover, under these conditions, lysolecithin had, not an inhibitory effect, but a slight stimulatory one. These results are shown by the experiment of figure 3.

Any attempt at a complete explanation of the phenomena described in the present paper must await a better understanding of the relationship between biotin and the fatty materials which can replace it in the nutrition of certain organisms. It seems most reasonable to suppose that biotin functions in the synthesis of these fatty materials (Williams, Broquist, and Snell, 1947). Lysolecithin might then behave as a true competitive analogue and in some manner block the synthesis. It could have no such effect if the products of the synthesis were supplied in the medium. In the light of the results with pantothenate and lysolecithin it would be necessary to extend the argument to assume that pantothenate also functions in the synthesis of the fatty materials. In any case it is interesting to note that lysolecithin has been observed to have a deleterious effect when fed to rats (Iwata, 1934). This effect was prevented by the inclusion of 3 per cent yeast in the diet.

SUMMARY

The growth of *Lactobacillus casei* could be completely prevented by the addition of low concentrations of lysolecithin to a medium which was complete except that it contained biotin in suboptimal amounts. With biotin concentrations up to 1 μg per tube, the amount of lysolecithin required to give complete inhibition varied directly with the concentration of biotin. If oleic acid or a fat-soluble biotin-active material from plasma was used in place of biotin, lysolecithin at concentrations up to ten times those found inhibitory with biotin had only a small growth-stimulating effect. In a medium containing excess biotin but suboptimal concentrations of pantothenic acid, growth of the organism was inhibited by appropriate concentrations of lysolecithin. Here again, if the biotin was replaced by an adequate concentration of oleic acid or the fat-soluble material from plasma, lysolecithin had a slight stimulatory effect rather than an inhibitory one.

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FURTHER STUDIES ON A FAT-SOLUBLE MATERIAL FROM PLASMA HAVING BIOTIN ACTIVITY

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A fat-soluble material from plasma has been found to have biotin-like activity for chicks as well as for lactic acid bacteria (1). Since oleic acid can replace biotin in the nutrition of *Lactobacillus casei* (2, 3) and of a variety of related bacteria (4) as well as in that of a yeast (5), it was of interest to determine whether oleic acid might also have a biotin-like activity for chicks. It is the purpose of this paper to report experiments concerned with this problem and to provide additional data on the biotin activity of the fat-soluble material from plasma, and on its occurrence and partial purification.

EXPERIMENTAL

General Methods—Biotin activity was determined by microbiological assay with *Lactobacillus casei* with the method of Landy and Dicken (6) slightly modified (1). Fat-soluble materials to be assayed were dissolved in warm ethyl alcohol, and distilled water was added to the solution to give a uniform emulsion.

The crude fat-soluble biotin-active material (FSF) was prepared in quantity by the ether extraction of acid-hydrolyzed horse plasma (1). 20 to 30 ml. of a neutral dark brown oil were ordinarily obtained from 10 liters of oxalated plasma. The activity of such preparations ranged from 1.5 to 3 γ of biotin activity per ml., and was usually about 2 γ .

All the chicks used were Rhode Island reds obtained when 1 day old. They were kept in electrically heated brooders until 10 to 14 days old, and then in wire bottom cages in a warm room. The stock diet was a chick starting mash of the following composition: yellow corn-meal 29, ground wheat 29, soy bean meal 17, alfalfa leaf meal 7.5, meat scrap 4.5, cod liver oil 0.7, charcoal 0.7, salt 0.7, calcite 2.1, fine grit 4.4, fine oyster shell 4.4. It contained about 13 γ of biotin per 100 gm. of diet. For the production of biotin deficiency, this mash was mixed with egg white, usually in the proportion of 20 parts of a commercially dried egg albumin in 100 parts of diet. A control casein diet contained in place of egg white washed casein mixed with riboflavin to provide 5 mg. of riboflavin per 100 gm. of casein.

The chicks were kept on the stock diet until they were 5 to 7 days old.

They were then placed on the experimental diets and the treatments, if any, begun. During the period of the experiment each chick received once weekly 3 to 4 drops of haliver oil with viosterol. The treatments consisted usually of the intramuscular injection of biotin, oleic acid, or FSF. The injections were given as 0.2 ml. of liquid in the breast muscle two times per week. The biotin was dissolved at appropriate concentrations in 0.85 per cent sodium chloride solution. The FSF and oleic acid were injected as the warm oils themselves. FSF was absorbed much better than oleic acid, but with both materials abscesses formed in the breast muscle surrounding small pockets of the oil.

At suitable intervals the experimental chicks were weighed and the severity of the dermatitis on the feet and at the corners of the mouth graded by a method similar to that of Ott (7), except that the scale used ranged from 0 to 6. Only occasional chicks, however, showed a dermatitis more severe than grade 4. In order to make this grading as nearly objective as possible, the chicks were taken at random from the various groups by an assistant and presented to the person doing the grading with their number tags covered. The latter individual then gave his judgment of the severity of the dermatitis to the assistant, who noted it beside the appropriate number. Thus the person grading the chicks could not be aware of the nature of the results until all the chicks had been graded. This precaution was desirable, since the experiments with FSF could not be performed in such a way as to give any likelihood of complete prevention of biotin deficiency symptoms. The maximal dosage of FSF which could be administered by injection was far from sufficient, in terms of its microbiological biotin activity, to be expected to provide complete protection from the deficiency. For this reason most of the experiments included two control groups injected with biotin, one with a dosage similar, on an activity basis, to that of the group receiving FSF and one with a dosage adequate (8) to prevent the deficiency entirely. Larger amounts of FSF could be administered by stomach tube, but it has not so far been possible to find any activity of the material when given by this route to chicks on an egg white diet.

Results

Treatment of Chicks on Egg White Diet with FSF and Oleic Acid—The effect previously reported (1) of FSF in mitigating the dermatitis produced in chicks by a diet high in egg white has been repeatedly confirmed. Tables I and II give the results of two experiments of this type, the usual commercial preparation of egg white having been used for one experiment and an acetone-precipitated preparation from fresh egg white (9) for the other. In Table I it will be noted that neither the group receiving FSF nor the groups receiving the lower dosage of biotin gained weight to any greater

extent than the untreated group on the egg white diet. Since this was also true in all the other experiments, the weights are not included in Tables II to V. It has been found by others (10) that lower dosages of biotin are required to affect the dermatitis than to permit normal weight gain in chicks.

Preliminary experiments with oleic acid revealed no effect on the dermatitis of chicks on an egg white diet. The results of two experiments com-

TABLE I

Effects of FSF and Biotin on Biotin Deficiency in Chicks Fed Diet High in Egg White

The chicks were started on the special diets and injections when 1 week old.

Diet	Injection (breast muscle)	Weekly dosage, mi- crobiological assay	No. of chicks	Average weight (gm.)					Average degree of derma- titis of feet and mouth		
				7 days	15 days	21 days	28 days	39 days	21 days	28 days	39 days
		<i>γ biotin</i>		gm.	gm.	gm.	gm.	gm.			
Casein, 20%	None		7	57	98	156	203	281	0	0	0
Egg white, 20%	"		7	58	87	133	163	205	3.4	3.3	4.9
	FSF	0.8	7	58	85	125	150	182	1.7	2.7	3.0
	Biotin	0.8	7	57	96	138	159	205	0.7	0.4	1.6
	"	6.0	6	55	88	140	182	251	0.7	0	0

TABLE II

Effect of Intramuscular Injection of FSF in Chicks on Diet containing 500 Gm. of Dried Acetone-Precipitated Fresh Egg White per 20 Pounds of Feed

Chicks placed on diet when 8 days old, injections begun 3 days later.

Treatment	No. of chicks	Average degree of dermatitis of feet and mouth	
		21 days	28 days
None.....	8	1.5	2.1
FSF 0.8 γ biotin activity per wk.....	9	1.0	1.5

paring FSF preparations with U. S. P. oleic acid and with a soy bean oil distillate (SBO) containing oleic acid¹ are shown in Tables III and IV. Although the oleic acid and the SBO had higher biotin activities for *Lactobacillus casei* than the FSF preparations, neither reduced the extent of the dermatitis, while both FSF preparations did so, one of them (from horse plasma, Table IV) to almost as great an extent as did a comparable dosage of biotin.

A single somewhat purified preparation of FSF has been tested in chicks

¹ The molecular distillate of soy bean oil was very kindly supplied by Dr. P. L. Harris of Distillation Products, Inc.

and found to be active (Table V). Table V also shows that FSF is active when injected into the posterior peritoneal cavity as well as when injected intramuscularly.

TABLE III

Intramuscular Injection of U. S. P. Oleic Acid, FSF from Human Plasma Fraction IV-1, and Biotin in Chicks Placed on Egg White Diet When 5 Days Old*

Injectons begun at 7 days of age.

Injection	Weekly dosage, by microbiologi- cal assay	No. of chicks	Average degree of dermatitis of feet and mouth			
			20 days	30 days	34 days	44 days
	<i>γ biotin</i>					
None.....	0	7	1.2	3.2	2.9	3.2
Oleic acid.....	1.2	8	1.3	2.7	2.9	3.6
FSF.....	0.6	8	1.0	2.8	2.2	2.6
Biotin.....	0.6	7	1.1	1.7	1.6	1.1
"	6.0	8	0.8	0.7	0.5	0.1

* A large supply of Fraction IV-1 from human plasma was obtained through the generosity of the American Red Cross.

TABLE IV

Comparison of Effects of Intramuscular Injection of Soy Bean Oil Distillate (SBO) containing Oleic Acid, of FSF from Horse Plasma, and of Biotin in Chicks on Egg White Diet

Diet and injections begun at 5 days of age.

Injection	Weekly dosage, by microbiological assay	Average degree of dermatitis of feet and mouth (10 chicks)		
		21 days	28 days	34 days
	<i>γ biotin</i>			
None.....	0	1.8	2.6	2.9
SBO.....	1.2	2.0	2.4	2.6
FSF.....	0.9	1.5	1.7	2.0
Biotin.....	1.0	1.4	1.3	1.6
"	10.0	0.4	0.1	0.2

Plasma and Tissue Levels of Biotin and Fat-Soluble Biotin-Active Materials

—The plasma levels of biotin and FSF in young ducks and chickens and the changes which they undergo during acute infections with avian malaria parasites have been previously described (11). The biotin content of the plasma of normal young ducks and chickens is about 2 to 4 mγ per ml., while the bound FSF content in terms of its biotin activity is about 10 to 15 mγ per ml. No bound biotin and relatively little free FSF have been found in the plasma.

Assays of the plasma of adult birds have shown that, while in males both the biotin and FSF remain much the same as in young birds, in females

TABLE V

Effects of Injection of FSF from Horse Plasma (Fraction A), of Fraction F Obtained from It by Adsorption on Aluminum Oxide and Elution with Ammoniacal Alcohol, and of Biotin in Chicks on Egg White Diet

Diet and injections begun at 5 days of age.

Injection	Weekly dosage, by microbiological assay	No. of chicks	Average degree of dermatitis of feet and mouth	
			19 days	26 days
	<i>γ biotin</i>			
None.....	0	5	1.7	2.8
Fraction F*.....	3.0	7	1.2	2.1
" A*.....	1.2	6	1.3	2.2
" A†.....	1.2	5	1.4	1.8
Biotin*.....	1.0	7	0.9	1.5
" *.....	10.0	6	0.6	0.4

* Injected to breast muscle.

† Injected to posterior peritoneal cavity.

TABLE VI

Biotin and FSF Content of Plasma of Adult Ducks (5 to 6 Months Old) As Determined in Two Different Ways

Duck		Biotin activity, mγ per ml. plasma					
No.	Sex	Free biotin		Bound biotin	Free FSF	Bound FSF	
		(a)	(b)	(c)	(d)	(e)	(f)
1	♀	23.3	29.2	0	2.2	34.0	28.1
2	♀	26.0	29.3	-1.3	3.2	38.8	35.5
3	♀	30.0	30.0	-2.5	4.5	31.5	31.5
4	♀	32.0	33.0	-1.8	1.5	27.8	26.8
5	♂	4.7	6.3	-1.7	1.1	17.7	16.1

(a) The activity of plasma diluted in water and shaken with ether. (b) The activity of plasma hydrolyzed in sulfuric acid minus the activity of the same preparation tested in the presence of 1:500 egg white. (c) The activity of plasma hydrolyzed in sulfuric acid and shaken with ether minus the free biotin (a). (d) The activity of plasma diluted in water minus the free biotin (a). (e) The activity of plasma hydrolyzed in sulfuric acid minus the activity of plasma diluted in water. (f) The activity of plasma hydrolyzed in sulfuric acid and tested in the presence of 1:500 egg white, minus the free FSF.

which are laying eggs both values are increased. Table VI gives a small representative sample of the data which have been obtained and at the

same time shows the good agreement between the values as determined by removal of FSF with ether and by inactivation of the biotin with egg white. Egg-laying hens showed a high biotin and FSF content of the plasma entirely similar to that of the egg-laying ducks. The increase in biotin may well be related to the high biotin content of the yolk of the egg, which in turn is essential for hatchability (12). In a similar way, avidin secretion by the oviduct accompanies egg-laying activity in hens (13). An increase in the total plasma lipides of egg-laying chickens has been demonstrated (14). Evidently the lipide or lipides with biotin activity share in this increase. It is noteworthy that the free FSF activity remains relatively low in the plasma of the egg-laying birds, most of the increase occurring in the FSF which is bound, presumably as a lipoprotein.

Much of this bound FSF can be precipitated by dilution of the plasma of egg-laying hens or ducks with distilled water in a ratio of 1:20. The protein so precipitated and collected by centrifugation redissolves readily in phosphate buffer of pH 7.4 to give a highly opalescent yellow solution. The bound FSF activity of such solutions, expressed as millimicrograms of biotin per mg. of protein, was $1\frac{1}{2}$ to 3 times as high as that of the original plasma.

Some determinations have been made of the distribution of biotin and fat-soluble biotin-active materials in tissues other than blood. The results for 5 to 6 month-old chickens are presented in Table VII. Since in the growth of *Lactobacillus casei* biotin can be replaced by oleic acid and a variety of related compounds, the microbiological assay of biotin activity due to fat-soluble materials from tissue would determine the sum of the concentration in the tissue of a number of substances. That oleic acid in depot fats did not enter appreciably into the measurements is indicated by the very low FSF activity of the abdominal fat, a tissue which contains about 40 per cent by weight of oleic acid combined as glycerides (15). Whatever the actual substances from tissues may be which have here been measured as FSF, it is remarkable that, except for the liver and kidney which have exceptionally high biotin contents, there is a general parallelism between the biotin content and the relative concentration of the fat-soluble biotin-active materials. A similar situation exists in egg yolk. For example, the yolk of one egg had a total biotin activity of 750 m γ per ml., of which 360 m γ remained after shaking with ether. The figures for biotin proper which are here presented are for the most part somewhat lower than those previously reported, though of the same order of magnitude. Thus Eakin *et al.* (16) found for the organs of a 10 week-old chicken 2600 m γ per gm. in the liver and 2500 m γ in the kidney, but only 65 m γ in the brain. Values for egg yolk have been found ranging from 400 to 600 m γ per ml. (17) with a usual value of about 500 m γ per gm. (12).

Partial Purification of FSF from Horse Plasma—Two different methods have yielded products having as high a specific activity (millimicrograms

TABLE VII

Biotin and FSF Contents of Representative Tissues from 5 to 6 Month-Old Chickens

Samples of the tissues were ground in saline in a glass grinder, autoclaved in 2.5 N sulfuric acid for 1 hour at 15 pounds, brought to pH 9.6, and diluted with water. The uniform suspensions thus obtained were divided into two portions, one of which was assayed for its total biotin content, while the other was shaken with three to four portions of ether and then assayed. The results of the latter assay gave the biotin content of the preparation, while the difference between the two assays was considered as activity due to FSF. In some cases, indicated by an asterisk, FSF activity was determined directly by the assay of suitable dilutions of the suspension in the presence of egg white.

The results are expressed as millimicrograms of biotin activity per gm. of fresh tissue.

Sex	Chicken No.	Brain	Lymph nodes	Breast muscle	Liver	Abdominal fat	Spleen	Oviduct	Testis	Adrenal glands	Kidney
♀	1	87† (165)‡	34 (83)	13 (43)	592 (129)*	7 (10)	45 (83)	80 (82)			993 (122)*
	2	107 (203)	57 (105)	15 (52)	1380 (160)*	2 (4)	72 (98)	134 (106)			1390 (110)*
	3	97 (195)	38 (77)	31 (40)	780 (131)*	6 (5)	55 (116)	179 (88)		172 (248)	1790 (74)*
	4	69 (160)	37 (75)	13 (35)	750 (102)*	4 (4)	52 (82)	53 (99)		112 (151)	805 (60)*
	5	142 (141)	49 (60)	29 (48)	1250 (75)	8 (15)	68 (102)	123 (79)			
	6	127 (191)	87 (106)	36 (41)	740	3 (2)	77 (89)	118 (61)		131 (133)	1150
♂	7	91 (168)	46 (65)	19 (35)	850 (62)*	15 (8)	42 (103)			105 (140)	895 (56)*
	8	87 (174)	187 (58)	10 (43)	605 (64)*		38 (99)			150 (136)*	1675 (72)*
	9	126 (189)	80 (101)	27 (39)	1220	13 (17)	64 (110)			170 (142)	2140
	10	82 (228)	44 (76)	18 (34)	1210 (60)	5 (4)	42 (88)			211	1625 (0)
	11		197 (100)	24 (42)	1040 (180)		91 (71)		66 (114)	86 (151)	1123 (162)
Averages...		101 (181)	78 (83)	21 (41)	947 (107)	7 (7)	59 (95)	114 (86)		142 (157)	1369 (82)

† The upper figures represent biotin throughout.

‡ The lower figures represent FSF throughout.

of microbiological biotin activity per mg. of dry weight) as commercial samples of U. S. P. or c.p. oleic acid. The products so obtained differed

obviously from oleic acid in being at ordinary room temperature (20–25°) whitish or pale yellow waxy solids.

One method was a crude counter-current distribution employing nine separatory funnels (18). Table VIII illustrates the results of such an experiment. The starting material in this case was prepared by fractionation in alcohol. 75 ml. of the oil from hydrolyzed horse plasma were suspended in 200 ml. of hot alcohol. The insoluble portion was extracted with two more portions of hot alcohol. The combined alcohol solutions were refrigerated overnight and filtered in the cold until clear. The portion soluble in cold alcohol, which contained virtually all of the activity, was concentrated *in vacuo* to remove the alcohol. A dark brown oil was obtained

TABLE VIII

Counter-Current Distribution of FSF Activity in Nine Separatory Funnels between Cyclohexane and 90 per Cent Alcohol

Funnel No.	Total activity for <i>Lactobacillus casei</i> when tested at concentrations of 1:2000 and less	Specific activity
	my	my per mg. dry weight
0	0	
1	420	
2	900	4.1
3	880	4.0
4	820	5.1
5	540	6.8
6	360	
7	60	
8	0	
Total recovered out of 3750 my...	3980	

which solidified in the cold. 1 ml. of the oil was dissolved in cyclohexane (previously shaken with 90 per cent ethyl alcohol) to a volume of 10 ml. This solution was then used for an eight plate distribution between cyclohexane and 90 per cent ethyl alcohol, each of which had been previously thoroughly shaken with the other solvent. A theoretical curve based on the distribution coefficient (about 1.1) of the biotin activity between cyclohexane and 90 per cent alcohol would have placed the maximal amount of activity in Funnel 4. Actually, it was distributed between Funnels 2 to 4. However, the highest specific activity occurred in Funnel 5. In another similar experiment the maximal amount of activity was distributed between Funnels 3 to 5 and the specific activity of the material from Funnel 4 was 10 my per mg. A sample of U. S. P. oleic acid dissolved in alcohol, which was assayed at the same time with this fraction and for which the dry weight

was determined in the same manner, gave a specific activity of 10.9 m γ per mg. However, in other assays the specific activity of oleic acid would be as low as 7 m γ per mg. It is important to note here that some fluctuation in the assay results is introduced as a result of the toxicity of higher concentrations of the fat-soluble materials. The toxic effect was relatively small with most of the preparations of the original oil from hydrolyzed horse plasma, so that at suitable concentrations nearly maximal growth was obtained. Moreover, with these crude FSF preparations the dose-response curve closely approached that with biotin itself (1). Such is not the case with oleic acid (2) or with the more highly purified preparations of FSF. Higher concentrations of these substances all exhibit a growth-inhibiting effect on *Lactobacillus casei*, before maximal growth has been attained.

TABLE IX

Chromatographic Adsorption of FSF from Alcoholic Solution onto Aluminum Oxide

Solvent passing through column	Fraction No. (each collected from bottom of column for 10 min. period)	Total biotin activity for <i>L. casei</i>	Specific activity
		m γ	m γ per mg. dry weight
95% ethyl alcohol	1, 2	0	
	3	10	
	4-8	0	
	9-12	0	
5% ammonia in 66% ethyl alcohol	13	15	
	14	45	9.0
	15	70	10.8
	16	60	9.2
Starting material.....		1920	3.2

The second method of partial purification depended on chromatographic adsorption on aluminum oxide and elution with ammoniacal alcohol. The activity could be adsorbed either from a cyclohexane solution or from an alcoholic solution containing only that portion of the original oil which was soluble in cold alcohol. When such a solution was allowed to percolate through a 10 \times 2 cm. column of aluminum oxide prepared in the corresponding solvent and was followed by more of the same solvent, an orange-yellow zone soon formed and migrated down the column at a fairly rapid rate. All the percolates appeared colorless, except those which included the zone and which were bright yellow. In repeated trials, the fraction containing the colored material has had a low but significant activity, whereas the fractions preceding and following it have had little or no activity. Most of the activity of the original solution, however, remained ad-

sorbed as long as cyclohexane, 95 per cent alcohol, or alcohol acidified with HCl was passed through the column. Elution began soon after the addition of ammoniacal alcohol (5 per cent ammonia in 66 per cent ethyl alcohol) and continued slowly over a considerable period. Tables IX and X give the results of two experiments of this type. In the experiment illustrated in Table IX, the yellow material was collected as a separate fraction (No. 3). It is obvious that in this experiment elution was still occurring at an appreciable rate when the collection of fractions was discontinued. The high specific activity of Fractions 14 to 16 is worthy of note. In the larger scale experiment illustrated in Table X the yellow material was not collected separately but it probably was responsible for most of the 460 m μ of activity

TABLE X

Chromatographic Adsorption of FSF from Alcoholic Solution onto Aluminum Oxide

Material	Total activity	Specific activity
	m μ	m μ per mg. dry weight
Starting material. Cold alcohol-soluble portion of oil from hydrolyzed horse plasma in 95% alcohol	20,000	
Combined percolates from sample and 100 ml. 95% alcohol	460	
1st hr.'s percolate after start of 5% NH ₃ in 66% alcohol	270	
Next 20 min. percolate	1,000	3.2
" 20 " "	1,000	5.4
" 20 " "	800	
" 20 " "	800	6.7
" 20 " "	800	6.7
Total recovered in percolates.....	5,130	

present in the combined percolate from the sample and the following 100 ml. of alcohol.

A larger quantity of a partially purified material was prepared by gross adsorption and elution. 95 ml. of the oil from horse plasma were dissolved in cyclohexane to 400 ml. 150 gm. of aluminum oxide were added and the mixture was stirred for half an hour. It was then filtered. To the filtrate were added an additional 100 gm. of aluminum oxide, and the mixture was again stirred for half an hour. It was filtered through the same paper which had been used previously, and the combined residues were washed with 300 ml. of cyclohexane. The combined filtrates were concentrated *in vacuo* at 45–50° to give 50 ml. of a brown oil with about one-fourth the activity of the starting material. The aluminum oxide residue was sus-

pended in 560 ml. of 5 per cent ammonia in 66 per cent ethyl alcohol and shaken mechanically half an hour. The aluminum oxide was allowed to settle and the supernatant was poured off through a filter paper. The aluminum oxide was then resuspended in an additional 280 ml. of ammoniacal alcohol and was again shaken mechanically for half an hour. The mixture was filtered. The combined clear amber filtrates were brought to a pH of 7 with 6 N HCl to give a cloudy liquid with droplets of brown oil. This was shaken in 100 ml. portions with 100 ml. of cyclohexane in a separatory funnel. The same cyclohexane was used for three portions of the neutralized eluate. All of the aqueous-alcoholic lower layers were pooled. On concentration of these *in vacuo* at 50° droplets of brown oil appeared when the volume was about 200 ml. This material was taken up in ether. On removal of the ether there were obtained 4 ml. of dark brown oil with an activity of 6.5 γ per ml. The combined cyclohexane extracts were concentrated *in vacuo* at room temperature to a small volume and left overnight. A light brown waxy material was obtained which, when warmed, became an easily flowing light brown liquid. 12 ml. of this material were obtained, with a biotin activity for *Lactobacillus casei* of 7.6 γ per ml. This was the fraction (F) which was found to have biotin-like activity for chicks (see Table V).

DISCUSSION

The experiments reported in the present paper support the conclusion that hydrolyzed plasma yields a substance, readily extractable with ether, which, when injected intramuscularly, has biotin-like activity in reducing the extent of the dermatitis produced in young chicks by a diet high in egg white. Usually the effect was not as great as that obtained by the injection of a comparable dosage of biotin in terms of microbiological biotin activity. Several explanations for this may be suggested. In the first place, while there was never any leakage of the injected saline-biotin solutions on withdrawal of the needle, some leakage of the oils with FSF activity frequently occurred. While the saline was rapidly and completely absorbed, this was not true of the oil, which moreover had some toxic properties. Finally, it is possible that the oil obtained from hydrolyzed horse plasma contains more than one substance with biotin activity for *Lactobacillus casei* but only one with biotin activity for chickens. This possibility is supported by the high biotin activity for *Lactobacillus casei* of oleic acid, a substance with which it has not been possible to demonstrate any biotin activity in chickens.

In the studies directed toward purification of the active material in FSF the activity was followed only by microbiological assay, since amounts sufficient for assay in chicks could not readily be obtained. It was hoped that

methods in which the material was subjected to relatively mild treatments would yield final products retaining their activity for chickens. This hope was realized with the partly purified fraction (F) obtained by adsorption and elution. More drastic procedures such as saponification were avoided, although in the first work concerning the fat-soluble biotin-active substance from hydrolyzed plasma it was found that the activity for *Lactobacillus casei* remained in the unsaponified fraction (1). Hofmann and Axelrod (19), in their note confirming the finding of a neutral oil from plasma with biotin activity, reported that the activity went into the saponifiable fraction. On the basis of personal communication, it would appear that this discrepancy is the result of a longer period of heating with alkali employed by the latter workers than by the former, who heated for 45 minutes. With several hours heating, the activity of FSF for *Lactobacillus casei* goes into the saponifiable fraction. Such saponified material has not been tested for its activity in chicks.

A number of authors have suggested ways in which oleic acid and related compounds might function in bacterial metabolism. Dubos (20) has concluded that in the growth of his *Micrococcus C* they probably act as catalysts, whereas in the growth of tubercle bacilli they enter into the synthesis of cell protoplasm. Guirard *et al.* (21) have noted their rôle as substitutes for acetate in the nutrition of some of the lactic acid bacteria. Still other lactic acid bacteria require oleic acid in addition to both biotin and acetate (4, 22, 23). Williams and Fieger (3) have postulated that both biotin and oleic acid function as cell permeability factors. Perhaps somewhat more likely is the hypothesis (4) that biotin is essential for the synthesis of oleic acid as it is for the synthesis of aspartic acid (24, 25).

The latter hypothesis could readily be applied to multicellular organisms, perhaps with the further addition that biotin enters into more than one stage of the synthesis of fatty compounds. It was early noted by Boas (26) that rats with severe biotin deficiency had almost no stores of body fat. Fatty infiltration of the liver and increase in body fat of rats have been observed after biotin administration (27), and more recently it has been found that rats with incipient biotin deficiency, unlike normal rats, did not store excess lipide in the liver when fed a diet high in cholesterol (28). The activity of the neutral oil from hydrolyzed horse plasma for chickens as compared to the inactivity of oleic acid for these animals suggests that perhaps in vertebrates the fatty acid must be supplied already combined in some manner in order to eliminate partially the need for biotin. It is also possible that the neutral oil is effective merely because it provides the fatty acid in less toxic form (4). In this connection it is interesting to note that for a quite different metazoan organism, the larva of the yellow fever mosquito, it has been found that, while both oleic acid and FSF can replace

biotin, the former material is active over a narrower range than the latter, presumably because of its greater toxicity.²

SUMMARY

The intramuscular injection of a fat-soluble material from hydrolyzed plasma into chicks fed a diet high in egg white reduced the severity of their dermatitis almost as much as did the injection of biotin in a comparable dosage in terms of microbiological biotin activity. The similar injection of oleic acid did not have such an effect, nor could the effect be produced by the oral administration of the material from plasma.

The concentration of the fat-soluble biotin-active material, as measured by microbiological assay in the plasma of ducks and chickens, shares in the general increase in lipides which occurs with the onset of egg-laying activity. The distribution of this material in the tissues of birds roughly parallels the distribution of biotin, except in the liver and kidney, which contain relatively very large amounts of biotin. Much of the bound fat-soluble biotin-active material in the plasma of egg-laying hens may be concentrated in a protein fraction precipitated by dilution of the plasma with water.

Partial purification of the active material from hydrolyzed horse plasma has been effected by counter-current distribution in separatory funnels and by chromatographic adsorption. Fractions have been obtained which have as high a specific activity for *Lactobacillus casei* as oleic acid but which differ from oleic acid in physical properties. A somewhat similar fraction prepared by gross adsorption and elution has been shown to have the biotin-like activity when injected into chicks on an egg white diet.

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THE RESISTANCE OF EGG-LAYING DUCKS TO INFECTION BY THE MALARIA PARASITE PLASMODIUM LOPHURAE

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Ducks have been generally considered to be highly susceptible, regardless of age and sex, to infection by the erythrocytic forms of *Plasmodium lophurae* (Wolfson, 1940; Hewitt, 1942). It was therefore surprising to find in 1942 several adult ducks which failed to develop any appreciable infection in spite of the fact that they had been inoculated intravenously with a large dose of heavily parasitized blood from ducklings. In the four resistant individuals which were first observed the parasites persisted in low numbers (50 to 200 per 10,000 red blood cells) for 3 weeks after inoculation. In smears made approximately every third day during this period, parasites of abnormal appearance became noticeable 3 to 10 days after inoculation and thereafter formed a progressively larger proportion of the small number of parasites present. Very few parasites with over two nuclei were ever seen, the very young trophozoites soon disappeared, and one to two nucleate trophozoites of normal appearance became less and less abundant. The abnormal forms were large and of two types, which may briefly be called "Dark" and "Light." The "Dark" forms showed after staining with Giemsa a dense blue cytoplasm, quite unlike the delicate blue usually seen in normal parasites. They had a large deep-pink nuclear mass. The "Light" forms showed a diffuse pale pinkish stain, suggesting a cell about to disintegrate. Although these two forms might at first be mistaken for female and male gametocytes, their pigment granules were small rather than coarse, as they are in the gametocytes of *P. lophurae*. The impression given by the blood smears was that in these 4 ducks the multiplication of the parasites was strongly inhibited. It soon became apparent that age was not the important factor, for several ducks even older than the 4 resistant ones were found to be fully susceptible. The observations were interrupted by the author's service in the Army. They were resumed in 1946 using a different strain of *P. lophurae* (obtained through the kindness of Dr. A. P. Richardson). With this strain an exact repetition of the earlier results has not been obtained, since the ducks used proved to be either highly susceptible or so resistant that they soon eliminated the infection, so that very few of the abnormal parasites were seen. It has, however, been possible to correlate resistance with egg-laying activity.

Methods

The strain of parasites was maintained at a high level by weekly intravenous passage in ducklings one to two weeks old.

The ducks were all White Pekins and were obtained as day-old ducklings. They were reared indoors until 6 to 8 weeks old, and thereafter in an outdoor pen

Frequency Distribution of Peak Parasite Numbers (as parasites per 100 red blood cells)

All ducks were 6 to 7 months old and were inoculated intravenously with enough parasites to give an initial count of about 1 per 100 red blood cells.

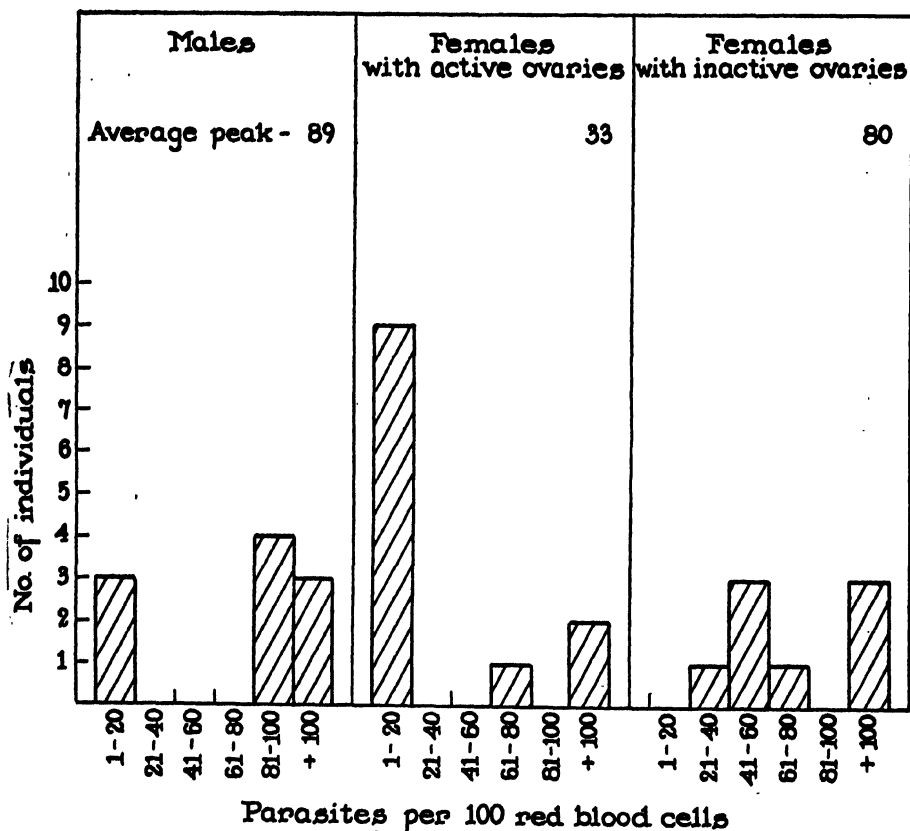


FIG. 1. A comparison of the density of parasitemia attained in female ducks with active and inactive ovaries and in males inoculated with the erythrocytic forms of *P. lophurae*.

with a screen bottom. They were fed a mixture of 50% ground whole wheat, 25% meat scrap, and 25% ground yellow corn. This was supplemented twice a week with lettuce or some other green vegetable. Some, but not all, of the female ducks began laying eggs when they were 6 to 7 months old. As groups

of 3 to 5 ducks reached this age, they were placed in individual indoor cages where their egg production could be readily followed. A heparinized blood sample (2 ml) was taken from some of the ducks and the plasma prepared for assay for its content of biotin and bound fat-soluble biotin-active material (Trager, 1947). The ducks were then inoculated intravenously with a dose proportionate to their body weight of heavily parasitized blood from an infected duckling. Enough parasites (one to 1.5 billion per kilogram body weight) were injected to give an initial parasite density of approximately one per 100 erythrocytes. Young ducklings, similarly inoculated as controls, invariably developed heavy infections. A thin blood smear, stained with Giemsa, was prepared from each duck within a minute after inoculation and once daily thereafter until death or recovery. These smears were used to determine the numbers of parasites per 100 red cells.

Birds which survived the infection were killed, and these, as well as the birds which died, were autopsied to confirm their sex, as determined by external characters, and to note the state of development of the ovary and oviduct or the testes.

RESULTS

Ten male and twenty female ducks were inoculated and observed in the manner described in the preceding section. The results are shown in Fig. 1. Of the 10 males, 7 developed very heavy infections, with peaks of over 81 parasites per 100 red blood cells. On the average, the peak parasite number occurred on the fifth day after inoculation. Among the females, 12 had active ovaries as shown either by the fact that they laid eggs shortly before inoculation or that, at autopsy, they showed a large oviduct and an ovary containing numerous developing yolks. The other 8 females did not lay eggs and, when autopsied, showed a small oviduct and a small ovary with no developing yolks. Of the 12 females with active ovaries, 9 had light infections, with a peak parasite number of 20 or less. In several of these the parasites underwent no increase in number whatever, so that the peak parasite number was the number present immediately after inoculation. The average time to reach the peak parasite number was only 3.4 days. In these resistant ducks, the phenomenon of long persistence of small numbers of parasites, consisting chiefly of abnormal forms, was not encountered. A few abnormal forms were seen, but in all the resistant birds the infections were rapidly cleared out. Of the 8 females with inactive ovaries 5 developed moderately heavy infections and the other 3 very heavy infections. The average peak parasite number was about the same as for the males. Similarly, the average time to the peak parasite density was 5.4 days.

The plasma concentrations of biotin and of the bound fat-soluble material, expressed in terms of its biotin activity, are shown in Table 1. It is evident that both the biotin and the lipoprotein (source of the bound fat-soluble

material) were higher in the plasma of female ducks than in that of males, and that both were higher in the plasma of females with active ovaries than in that of females with inactive ovaries.

TABLE I

The Concentrations of Biotin and Bound Fat-soluble Biotin-active Material (FSF) in the Plasma of 6 to 7 Month Old Ducks

Type of duck	No. of animals assayed	Biotin mγ/ml		Bound FSF as mγ of biotin activity per ml	
		Range	Average	Range	Average
Male.....	7	5-13	7	18-21	19
Female with active ovary.....	9	13-32	25	20-39	29
Female with inactive ovary.....	4	9-25	17	18-24	21

DISCUSSION

There can be no doubt that some physiological characteristic ordinarily associated with egg formation in the duck is responsible for the transformation of this animal otherwise highly susceptible to infection by *P. lophurae* into one highly resistant. The few females with active ovaries which nevertheless developed heavy infections, and the few resistant males, serve to emphasize the fact that individual differences in susceptibility occur which are quite unrelated to egg formation. In most of the ducks, however, these individual variations are overshadowed by the characteristic associated with egg formation.

Other work (Trager, 1947) has indicated that a plasma lipoprotein plays a rôle in the resistance of birds to avian malaria parasites. In keeping with this hypothesis is the fact that the lipoprotein content of the plasma of ducks with active ovaries is higher than that of ducks with inactive ovaries. Riddle and Burns (1927) have previously shown a much higher concentration of lipides in the blood of female ring doves during the ovulation cycle than in males, or females in the resting stage. Lorenz, Entenman and Chaikoff (1938) have demonstrated the same situation in more detailed fashion for the chicken. They have further shown that the increase in lipides can be produced by the injection, into immature male as well as female birds, of estrogenic hormones (Entenman, Lorenz and Chaikoff, 1940). These facts suggest the possibility of an indirect hormonal effect, via the lipoprotein of the plasma, on resistance to *P. lophurae*. It is worthy of note that estradiol benzoate has been observed to produce a mild lipemia in rats on a diet high in fat (Loeb, 1942), and that urinary estrogens increased the bactericidal effects of the blood serum of male rabbits on *Eberthella typhi* (Tsao, 1941).

Bennison and Coatney (1948) have recently reported a small effect of sex of the host on *Plasmodium gallinaceum* infection in very young chicks. Female

chicks 6 to 8 days old showed slightly higher densities of erythrocytic parasites, a poorer response to treatment with quinine, and earlier appearance of exoerythrocytic forms in the brain than did males. A more striking effect of sex on the course of a protozoan infection, and one perhaps more nearly analogous to the results reported in the present paper, is that described by Hauschka (1947) for *Trypanosoma cruzi* in mice. When brother and sister mice were inoculated in a comparable manner with *T. cruzi* the infection in the males attained parasite densities twice as great as those seen in the females.

SUMMARY

Ten male and twenty female White Pekin ducks 6 to 7 months old were inoculated intravenously with large doses of *Plasmodium lophurae*. In 9 of the 12 female ducks with active ovaries at the time of infection, the parasites underwent little or no multiplication. All 8 of the females with inactive ovaries developed heavy infections, as did 7 of the 10 males. The average free biotin and bound biotin-active lipid material of the plasma before inoculation were highest in the egg-laying females, lowest in the males and intermediate in the females with inactive ovaries.

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LEPTOSPIROSIS IN CATTLE*

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PLATES 10 TO 12

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During a study of bovine mastitis in groups of Holstein, Guernsey, and Brown Swiss dairy cows in April, 1946, three animals were observed whose milk had a bloody or thickened yellowish appearance. The incidence of this condition increased markedly in May and occasional cases have continued to occur. The affected cows showed fever and rarely hemoglobinuria; a few showed inappetence and a lowered milk yield.

The clinical manifestations and incidence indicated an infectious disease, and since bacteriological examinations of the milk revealed a mixed bacterial flora of no apparent etiological significance, attempts were made to transmit an infectious agent to laboratory animals.

Transmission Studies in Laboratory Animals

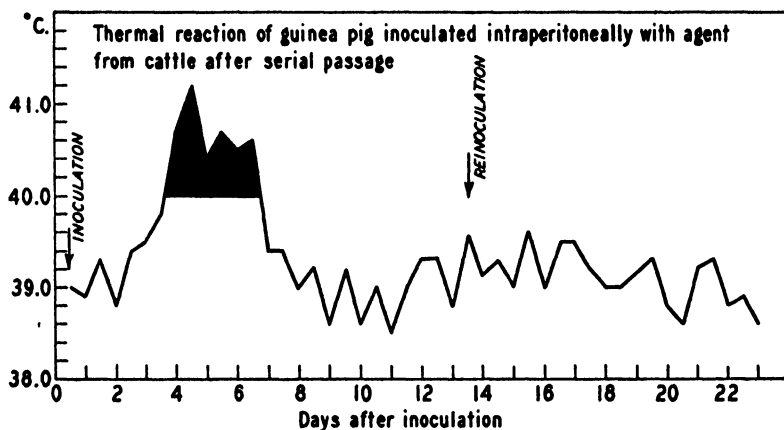
Guinea Pigs.—A stock of apparently healthy guinea pigs was moved into a constant temperature room which was maintained at 70°F. with 60 per cent humidity. Normal animals kept under these conditions never showed rectal temperatures exceeding 40°C. and readings above this were interpreted as fever. Groups of 2 or 3 guinea pigs weighing 250 to 350 gm. were each inoculated intraperitoneally with 1 to 5 cc. of freshly drawn abnormal milk. Thereafter rectal temperatures were taken twice daily on all animals and daily weighings made on selected groups. Defibrinated blood, obtained by heart puncture from guinea pigs showing fever, was inoculated into additional guinea pigs, as had been done for the original passage, with the exception that the size of animals varied from 150 to 600 gm.

A pathogenic agent was recovered from each of 5 specimens of abnormal milk, 3 of which were taken at the height of the outbreak (C161 on May 27, 1946, C162 on June 19, 1946, and C164 on June 19, 1946), another at the end of the outbreak (C168 on August 6, 1946), and the last from a single sporadic case approximately 3 months later (C181 on December 3, 1946). This agent when inoculated into guinea pigs and in animals of all sizes regularly produced febrile reactions which began 3 to 5 days after inoculation and lasted 2 to 4 days

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(Text-fig. 1). These effects were observed consistently for 3 strains carried through 5 serial passages, for another through 25 passages, and for still another through more than 50 continuous transfers. No additional signs of infection were noted, with the exception of a slight loss of weight during the febrile period which never exceeded 10 per cent of the total body weight. Five guinea pigs recovered from infection with each strain were given second inoculations with the homologous strain, and all of these animals proved immune. Similar groups of 5 guinea pigs each were then inoculated with different strains in complete reciprocal immunity tests. Each strain immunized against all of the others.

Guinea pigs killed during the febrile period showed on autopsy scattered petechial hemorrhages in the lungs and minute white spots in the liver. His-



TEXT-FIG. 1.

topathological examination of the lungs revealed alveoli filled with extravasated blood (Fig. 1). Liver lesions represented areas of liver cell necrosis midzonally located (Fig. 2); while sections of adrenals, spleen, heart, and kidneys showed no evidence of infection. Other guinea pigs killed after recovery showed essentially negative autopsy findings, with pigmented areas in the lungs as the only evidence of previous lesions.

Two uninoculated guinea pigs were placed in the same cage with 2 others each of which had been given intraperitoneally 1 cc. of defibrinated blood from infected guinea pigs. The two inoculated animals were allowed to develop illness and to recover before their removal from the cage. The contact animals after an observation period of 14 to 21 days failed to show signs of illness. They were then given the usual inoculation in a test for immunity. None were immune. This experiment was repeated 4 times with consistent results, which indicated that in guinea pigs the disease did not spread by contact.

Results obtained thus far indicated that an infectious agent had been secured in guinea pigs. Inoculation with this agent regularly caused definite signs of illness and lesions in the animals, regardless of size, and the disease did not spread by cage contact. Also, the infection resulted in complete immunity. A test for the agent therefore consisted of an initial intraperitoneal inoculation of guinea pigs and an observation period of 14 days, followed by a challenge inoculation with an established strain. This test was used in all of the work that follows.

Eggs.—In attempts to transfer the agent to 8, 9, or 10 day embryonated eggs inoculations were made on each chorioallantoic membrane with 0.1 cc. of blood from infected guinea pigs. Serial transfer was conducted by inoculating as for original passage with either a 10 per cent suspension of chorioallantoic membrane in saline or chorioallantoic fluid. In all, 3 strains were tested in this manner. After 10 serial passages tests with each strain showed that the agent was present in 10 per cent suspensions of chorioallantoic membrane in saline, in 10 per cent embryo suspension in saline, and in chorioallantoic fluid. Serial transfers were continued with one strain through 50 passages and subsequently chorioallantoic fluid was inoculated into guinea pigs and calves. Typical signs and lesions were produced. As an additional test guinea pigs recovered from infection produced with this egg passage material were inoculated with the agent that had been maintained for more than 50 continuous transfers in guinea pigs. These animals proved immune. Inoculated eggs showed no definite signs of infection in early passages (1st to 10th) but thereafter most embryos died 7 days after inoculation, although they appeared to develop normally for 5 days.

Mice.—Each of a group of 5 mice was inoculated intraperitoneally with 1 cc. of infected chorioallantoic fluid that had been transferred serially for 8 passages. Five days after inoculation the mice were autopsied. A 10 per cent suspension of pooled spleens was prepared and inoculated into another group as described above. The presence of the agent could be demonstrated after 5 serial passages in mice although all mice remained apparently normal and no lesions were found on autopsy.

Rabbits.—Each of 2 rabbits was inoculated with 1 cc. of infected chorioallantoic fluid that had been serially transferred for 8 passages. Five days after inoculation 5 cc. of blood was removed by heart puncture from each rabbit, pooled, and 2 other rabbits were inoculated with it as described above. After 5 serial passages tests showed the agent present in the blood of the inoculated rabbits. Temperature reactions similar to those obtained in guinea pigs were shown by all inoculated rabbits in each passage. Autopsies made during the febrile period on one rabbit from each series disclosed no gross lesions.

Miscellaneous Animals.—Inoculations were made subcutaneously with 1 cc. chorioallantoic fluid from infected eggs into each of 3 puppies (2, 4, and 8 weeks

old) and into 1 pig weighing 30 pounds. None of these animals showed increased temperature or other signs of illness. Each of a group of 6 hamsters was inoculated intraperitoneally with 1 cc. of defibrinated blood from infected guinea pigs; the strain used had been serially transferred through 89 passages. Of this group 3 died 9 to 12 days after inoculation. Autopsies showed hemorrhages in the lungs. The remaining hamsters showed pale shrunken kidneys when killed 25 days after inoculation. Similar results were obtained from another group of hamsters inoculated in the same manner with material that had been serially transferred through 12 passages. Tests for the infective agent were not made on any of these animals.

Nature of the Etiological Agent

Properties.—The greatest concentration of the agent was found in the chorioallantoic fluid from infected eggs 5 to 7 days after inoculation and this never exceeded 10,000 infective doses per cc. for guinea pigs. Using material of this sort, tests showed that the agent did not survive lyophilization and that freezing with dry ice caused at least a thousandfold loss in activity, while continued storage for 6 months under dry ice refrigeration resulted in complete loss of activity. Centrifugation at a speed of 24,000 R.P.M. did not remove the infective agent from the supernatant fraction of the chorioallantoic fluid from infected eggs. In 2 separate tests of this latter, the agent was found to pass through Berkefeld N filters.

All attempts failed to demonstrate a cultivable agent in defibrinated blood from infected guinea pigs, or in chorioallantoic fluid from infected eggs, by inoculation of blood agar slant and sealed cooked meat medium incubated at 37°C. Additional cultures made of chorioallantoic fluid from infected eggs in Fletcher's medium showed no growth when incubated at room temperature (approximately 22°C.). Films of defibrinated blood from infected guinea pigs and chorioallantoic fluid from infected eggs stained by methylene blue, Gram's method, Giemsa's method or Macchiavello's method showed no visible forms.

Demonstration of Spirochetes.—Preparations of blood and kidneys from infected guinea pigs infective for other animals showed no organisms upon dark-field examination ($\times 160$). We are indebted to Dr. J. B. Nelson for staining the preparations of chorioallantoic fluid from infected eggs by Morosow's method, thus permitting the first demonstration of spirochetes. Later dark-field examinations of chorioallantoic fluid from some infected eggs showed in each field 3 to 5 spiral-shaped organisms that rotated rapidly on the long axis. In general, organisms were more readily found in eggs 5 days after inoculation and before death of embryos than 7 days after inoculation or following death of the embryos. A few forms were found also in blood from calves inoculated with chorioallantoic fluid from infected eggs. Examination of the urine from these calves showed no organisms although it was capable of infecting guinea pigs.

Sections of kidneys from 5 calves inoculated with either chorioallantoic fluid from infected eggs or defibrinated blood from infected guinea pigs were stained by Levaditi's method but showed no organisms. Films of blood and kidneys from infected guinea pigs stained by Morosow's method showed no organisms. Preparation of chorioallantoic fluid from infected eggs or cultures similarly treated showed a few spiral-shaped forms (Fig. 3).

Chorioallantoic fluid from infected eggs, blood from infected guinea pigs, blood from infected rabbits, blood from experimentally infected calves, and urine from experimentally infected calves after centrifugation at 10,000 R.P.M. for an hour were inoculated into tubes of semisolid meat infusion agar that contained either 10 per cent rabbit, calf, or horse serum. These cultures then were placed at 37°C., 30°C., or room temperature (approximately 22°C.). Darkfield examinations made 3 days later showed a few spiral-shaped organisms in the tubes incubated at 37°C. After 2 weeks, examination of tubes placed at 37°C. revealed a diffuse growth in the top portion of the medium. Growth occurred also at 30°C. although it was more slow and required a month for similar development. No visible growth occurred at room temperature.

The spiral-shaped organisms growing in culture were examined with the electron microscope. Measurements showed a diameter of 90 millimicrons and a length, commonly of 4 microns (Fig. 4), but varying from 4 to 16 microns according to the number and depth of spirals. No internal structure was revealed.

Cultures of suspensions of organisms heated at 50°C. for 10 minutes showed no growth while those heated at 45°C. for 10 minutes grew. In 2 attempts, growth was obtained from Berkefeld N filtrates of a suspension of organisms. A suspension of previously motile organisms showed no activity after freezing with dry ice. Five guinea pigs which had been inoculated with 1 cc. of cultured organisms transferred 5 times in media were reinfected 14 days later with 1 cc. of chorioallantoic fluid from infected eggs. All were immune. Similarly 5 guinea pigs that had recovered from an infection produced with the agent maintained in eggs were immune when tested with cultured organisms.

Since the spirochete recovered in culture possesses physical and immunological properties similar to the infective agent, it appears legitimate to assume that it came from the sick cows originally studied.

The Experimental Disease in Cattle

Production.—Many of the cattle used came from an experimental herd maintained by this department. Repeated observations on these animals showed no evidence of this or any other infection. Some animals were purchased locally and observed for at least a week before use. In all, 5 lactating cows and 18 calves of Brown Swiss, Guernsey, Jersey, and mixed breeds were used.

Three of the cows were in their first milking period while the other 2 had had multiple lactations. The calves ranged in age from 8 days to 3 months. All

experiments were conducted upon single animals kept in strict isolation unless contact experiments were planned. Daily temperatures were taken for a week before inoculation in order to obtain an indication of each animal's normal range.

Various methods were used in attempts to produce the experimental disease: (a) subcutaneous injection of 1 cc. of defibrinated blood from infected guinea pigs (1 to 40 passages), (b) subcutaneous injection of 1 cc. of chorioallantoic fluid from infected eggs (5 to 50 passages), (c) subcutaneous injection of 1 cc.

TABLE I
Production of Disease in Cows and Calves

Method of inoculation	Inoculum	No. of animals inoculated*	Results	
			No. of animals showing infection†	No. of animals showing signs of illness
Subcutaneous	Blood from infected guinea pigs	5 { 2 cows 3 calves	2 3	5
	Chorioallantoic fluid from infected eggs	5 { 1 cow 4 calves	1 4	5
	Culture of spirochete	5 calves	5	5
Intranasal	Chorioallantoic fluid from infected eggs	5 calves	5	3
Fed	Chorioallantoic fluid from infected eggs	2 calves	0	0
Contact		5 { 3 cows 2 calves	2 1	0

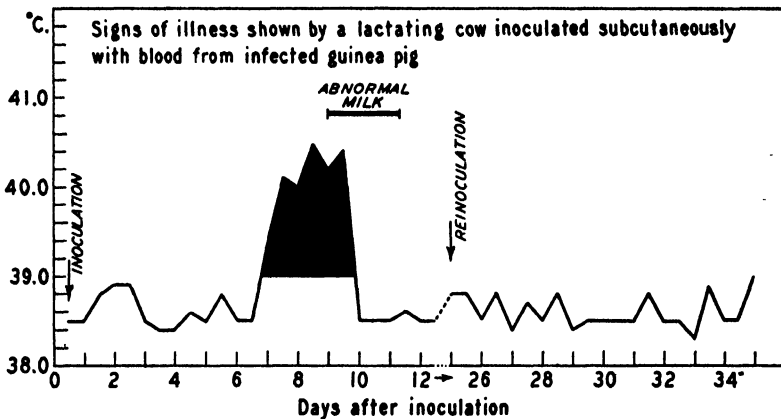
* Four animals counted twice.

† Infection based on signs of illness, recovery of infective agent, and/or immunity to subsequent injection subcutaneously.

of semisolid culture medium that contained spirochetes serially transferred for 1, 2, 5, 6, or 7 passages, (d) intranasal injection of 10 cc. of chorioallantoic fluid from the 6th to 16th serial passage in eggs, and (e) feeding of 25 cc. of chorioallantoic fluid from the 10th to 40th serial passage in eggs mixed with 1 liter of milk. Infectivity of all inocula was checked by inoculating 1 cc. intraperitoneally into a guinea pig. Contact experiments were made by placing normal animals in pen contact with infected ones. All animals were checked daily for increased temperature or other signs of illness. Animals which remained apparently normal were given a second inoculation subcutaneously with material from either infected guinea pigs or eggs. These results are summarized in Table I.

As can be seen in Table I, all 15 animals inoculated subcutaneously showed signs of illness. Three of 5 calves showed signs of illness following intranasal inoculation. The other 2 showed no signs of illness but were immune to a second inoculation given subcutaneously. Likewise 3 of 5 contact animals showed no signs of illness following exposure but subsequently were immune to subcutaneous inoculation, indicating that in these instances inapparent infection must have occurred. The infection was not transmitted in 2 contact and in 2 feeding experiments.

Features of the Illness.—Two lactating cows inoculated subcutaneously showed fever after an incubation period of 7 to 9 days. The findings in one of these animals are given in Text-fig. 2. Although these cows under experimental



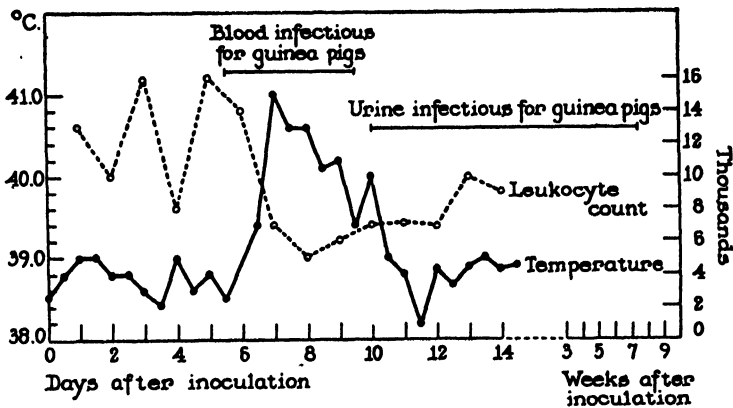
TEXT-FIG. 2.

conditions had low milk yields, the amount of milk was decreased further during the febrile period to approximately one-half that obtained before inoculation. In addition the milk became thickened, yellow in color, and contained flakes that represented collections of leucocytes. Blood as observed in natural cases could not be seen in any of the milk samples but in other respects the samples resembled those obtained from naturally infected cows. In contact experiments 2 cows placed with infected suckling calves showed no signs of illness or alterations in the milk although one animal later proved to be immune. Another cow placed in contact with naturally infected animals also showed no signs of illness or alterations in the milk but developed immunity.

Among 18 calves infected by subcutaneous or intranasal inoculation or by contact, 3 showed no signs of illness, 12 developed fever only, while 3 showed a febrile reaction and later died. Except for those with inapparent infections, a marked increase in temperature occurred 2 to 7 days after inoculation and lasted from 1 to 3 days. During the febrile period a slight anorexia was noted. All animals appeared normal following termination of fever except those that

later died. These animals became rapidly and progressively worse, showed a subnormal temperature, and died within 1 to 2 days. A hemoglobinuria signaled the approach of death.

For additional studies, specimens of blood from 5 animals were examined for total red cells and leucocytes before, during, and after illness. At the same time urine was collected and tests were made for albumin and microscopic examination of centrifuged sediment. These results are shown graphically in Text-fig. 3 for a calf and are typical of those studied. All calves showed a mild leucopenia during the febrile period. On the day that the temperature became normal, the urine showed albumin and large numbers of leucocytes which were found in the centrifuged sediment.



TEXT-FIG. 3. Features of illness shown by calf C188 inoculated intranasally with chorio-allantoic fluid from infected eggs.

Pathology.—At the end of fever 7 calves were examined: 3 that died from the infection and 4 that were killed. All organs seemed normal except the kidneys which appeared congested. Perirenal edema was noted in 2 calves. In addition the kidneys of 4 calves showed small white spots diffusely scattered over the surface. These spots, frequently irregular in outline, were approximately 1 mm. in diameter and appeared continuous with the normal surface. Section showed these spots extending either as wedges or as plugs into the cortical layer. The medullary portion appeared normal. The bladder and its contents were not unusual except in the animals that died. Collections of reddish colored urine were noted. One lactating cow and 6 calves that showed signs of illness were held for 14 days, given a second inoculation, and then killed after a further observation period of 14 days. Autopsies showed lesions in the kidneys similar to those seen in the early stages of the disease. Two calves that showed signs of illness were held 60 days after inoculation, then killed and

examined. White spots were found in the kidneys of one animal while the other showed no lesions.

A group of one lactating cow and 3 calves that showed no signs of illness was killed 14 days after a second inoculation. Autopsies showed no lesions.

A similarity of lesions in the kidneys among cows and calves in all stages of infection was found on histological examination. Widespread but focal involvement of the cortex and corticomedullary junction (Fig. 5) was noted and consisted of irregularly shaped patches of infiltrating cells predominantly mononuclear but mixed with varying numbers of eosinophilic polymorphonuclears (Fig. 6). Most tubules were destroyed in these patches. Tubules in the affected areas and at some distance away frequently contained masses of cells identical with those forming the infiltrates. Near the patches of cellular infiltrates mononuclear cells were seen between tubules in areas normally occupied by peritubular capillaries. Glomeruli in general appeared normal with patent vascular coils, but there was some evidence of capillary damage indicated by deeply staining eosinophilic nuclei and leakage of protein into capsular spaces. Tubules throughout the cortex were generally well preserved except in areas of cellular infiltration. Tubular lumina, however, contained in many cases protein precipitates. Here and there proximal tubules showed smaller cells without nuclei and with a finely vacuolated cytoplasm. The distal and intermediate tubules seemed less affected. There were no significant changes in collecting tubules.

Presence and Persistence of Spirochetes in Inoculated Animals.—Three cows were inoculated subcutaneously, 2 with blood from infected guinea pigs and the other with chorioallantoic fluid from infected eggs. Tests made at the time of inoculation and during the subsequent febrile period showed the presence of the agent in both blood and milk during the febrile period in one of the cows that had been inoculated with blood from infected guinea pigs. The agent was not recovered in either blood or milk from the other 2 cows. In similar tests made on blood, milk, and urine from a naturally infected cow during the acute phase of illness, the agent was recovered from milk samples only.

Tests made on all calves during the febrile period showed the agent in blood. From 2 calves, one inoculated subcutaneously with 1 cc. of defibrinated blood from an infected guinea pig and the other inoculated intranasally with 10 cc. allantoic fluid from infected eggs, tests for the agent were made daily on blood and urine from the time of inoculation through the febrile period and at 2 week intervals thereafter. These results, presented graphically in Text-fig. 3 for one calf, showed the agent present in the blood 1 day before the onset of fever and during the febrile period. On the day the temperature became normal, the blood no longer contained the infective agent which, however, persisted for 53 days in the urine. The other calf responded in a similar fashion. Additional studies on 3 calves during the incubation period, the febrile period, and a few

days thereafter showed similar results in the shift of the infective agent from the blood and its subsequent appearance in the urine.

Immunity Phenomena.—Tests for acquired immunity were made 14 to 21 days after subcutaneous or intranasal inoculations of 10 animals with blood from infected guinea pigs or chorioallantoic fluid from infected eggs. Each animal was reinfected subcutaneously with 1 cc. of either blood from infected guinea pigs or chorioallantoic fluid from infected eggs. The infectivity of the inocula was checked by simultaneous inoculation of guinea pigs with 1 cc. intraperitoneally. In all instances recovered animals showed no signs of illness after test inoculations, indicating immunity.

Inactivation of the spirochete was tested by intraperitoneal inoculation of guinea pigs with 2 cc. of a mixture of equal parts of sera of cows recovered from natural and experimental infection or calves recovered from experimental infection and semisolid culture medium that contained organisms. Both acute phase and preinoculation sera were tested. Sera from recovered animals completely inactivated the spirochetes whereas sera from animals during the acute phase of illness or before inoculation did not. In addition to animal tests, the effect of sera on suspensions of spirochetes was also observed under darkfield conditions. Preinoculation sera from experimental animals or acute phase sera from naturally infected animals had no observable effect on the spirochete when mixtures containing fresh guinea pig serum (complement) were observed after incubation at 37°C. for 2 hours. In similar tests sera from animals recovered either from natural infection or from experimental disease caused disappearance of the spirochetes presumably by lysis.

DISCUSSION

In a preliminary report a natural outbreak of an infectious disease of dairy cattle was attributed to a virus (1). An agent had been recovered which reproduced the disease and which resembled in several respects the Fort Bragg fever virus in humans reported by Tatlock (2) and the third virus of swine reported from Korea by Ochi and Miyairi (3). Most striking were the similarity of the experimental infection in laboratory animals and the loss of virulence of the agent lyophilized or stored at -50°C . On the other hand, some of the obvious signs and pathological features, bloody milk and nephritis in particular, resembled the leptospiral infections in cattle reported by Michin and Azinow (4) in Russia; Semschow (5) in Russia; Terskikh (6) in Russia; Bernkopf, Olitzki, and Stuczynski (7) in Palestine; Ungar and Bernkopf (8) in Palestine; and Freund (9) in Palestine. All these workers not only demonstrated the presence of leptospira in stained tissue sections of the liver and kidneys of affected animals but also showed by pure cultures and serological and animal inoculation studies that the leptospira was the etiological agent. On the other hand, in the outbreaks reported by Jungherr (10) in Connecticut, Marsh (11) in Mon-

tana, Johnson (12) in Australia, and Mathews (13) in Texas, the diagnosis was made after autopsy by staining tissue sections of diseased cattle to identify the leptospira. Certain clinical types of these leptospiral infections appeared identical with the natural disease observed in this outbreak but differed in most cases. Most significant was the fact that no abortion or icterus occurred and hemoglobinuria was rare although these features as well as the presence of leptospira in the kidney were reported by other workers. Since in our preliminary studies we were unable to demonstrate leptospira microscopically in kidney sections or culturally by the usual means of cultivation in Fletcher's medium at room temperature and since there were additional differences as well, further search was made for the etiological agent.

It was shown that the agent is a spirochete readily transmissible to guinea pigs, rabbits, embryonated eggs, mice, lactating cows, and young calves. Experimentally produced infection in the natural host resulted in a variation of obvious findings that ranged from a few cases of inapparent infection with normal urine through the usual condition of fever with albuminuria to occasional hemoglobinuria and death. These manifestations may explain the observations in the original outbreak of instances of hemoglobinuria and also the occasional animal in contact with natural cases that developed immunity without evidence of disease. The spirochete is not confined to the mammary gland but causes a generalized infection with subsequent localization in the kidney. In this organ the lesions and the infectious agent persisted for nearly 2 months, long after the latter had disappeared from the blood and after immunity had become established.

The following facts have a bearing on the spread of this disease. There is strong reason to believe that some animals undergo a form of natural nasal inoculation. Urine that contains the spirochete may be excreted from a standing animal onto a concrete barn floor, thereby causing a spray of droplets some of which in turn could be inhaled by nearby animals. This mode of infection might explain how a single animal could initiate the disease in a susceptible herd. This could have occurred in the outbreak herein reported, since the usual method of herd replacement was followed by constantly bringing in large numbers of animals from various regions of the United States.

The leptospiras from cattle found in Russia, Palestine, and Australia have been reported to cause disease in man. This has not been shown for the spirochete studied in the present paper but, since this organism is present in milk, infection might occur through ingestion, although experimental calves did not become infected by this route.

The agent was present in the blood during early stages and infection was produced by inoculation subcutaneously. Insect vectors therefore must be considered as a possible epidemiological factor and a search should be made for the reservoir hosts.

The organism that we have isolated stains with difficulty, is aerobic, shows a typical motility, often has hooked ends and therefore appears to be a leptospira (Bergey, 14). The disease it produces differs from other spirochetal diseases of cattle in that an abnormal milk is the dominant finding. Jaundice has not been seen and hemoglobinuria is very infrequent. As in most other spirochetal diseases lesions in the kidneys are found. The spirochetes were demonstrated with difficulty. Serological and other comparisons will have to be made before it can be determined whether this form has been described before. We would like to emphasize that these spirochetal infections are not easy to differentiate from virus diseases.

SUMMARY

From abnormal milk of cows an agent has been transmitted to guinea pigs, rabbits, mice, and embryonated eggs. This agent caused a febrile reaction in guinea pigs and rabbits and an inapparent infection in mice. In early passages embryonated eggs were unaffected but later death of embryos occurred 7 days after inoculation. When blood from infected guinea pigs or chorioallantoic fluid from infected eggs was inoculated subcutaneously or intranasally into young calves, fever with albuminuria and more rarely hemoglobinuria was produced, in lactating cows the infection resembled that seen in animals with natural disease. Pen contact of normal cows and calves with infected calves resulted in inapparent infection. Autopsies showed that in addition to causing altered milk secretion, the agent damaged the kidneys and produced an interstitial nephritis. The agent was recovered from blood and milk during the febrile period and was demonstrated in the urine for periods long afterwards. Antibodies for the spirochete were found in the sera of experimental animals and of cows recovered from the natural disease.

The blood of infected guinea pigs, the chorioallantoic fluid from infected eggs, and the blood or urine from experimentally infected calves yielded a culture of a spirochete which appeared identical with the infective agent in comparative tests of physical, pathogenic, and immunological properties.

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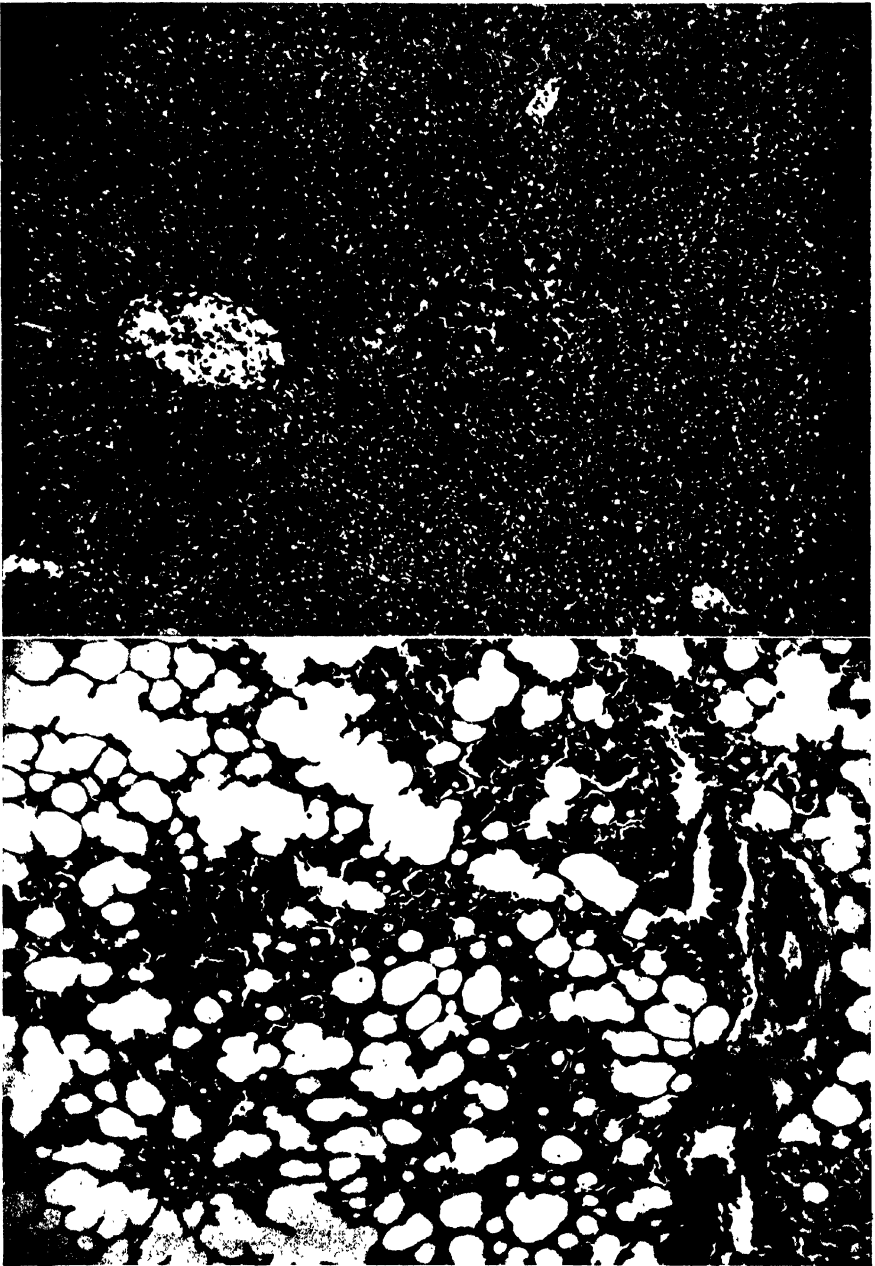
EXPLANATION OF PLATES

PLATE 10

Photographs by Mr. J. A. Carlile.

FIG. 1. Lung from infected guinea pig showing hemorrhage into alveoli. Stained with polychrome methylene blue. $\times 112$.

FIG. 2. Liver from infected guinea pigs. Note area of necrosis. Stained with polychrome methylene blue. $\times 112$.

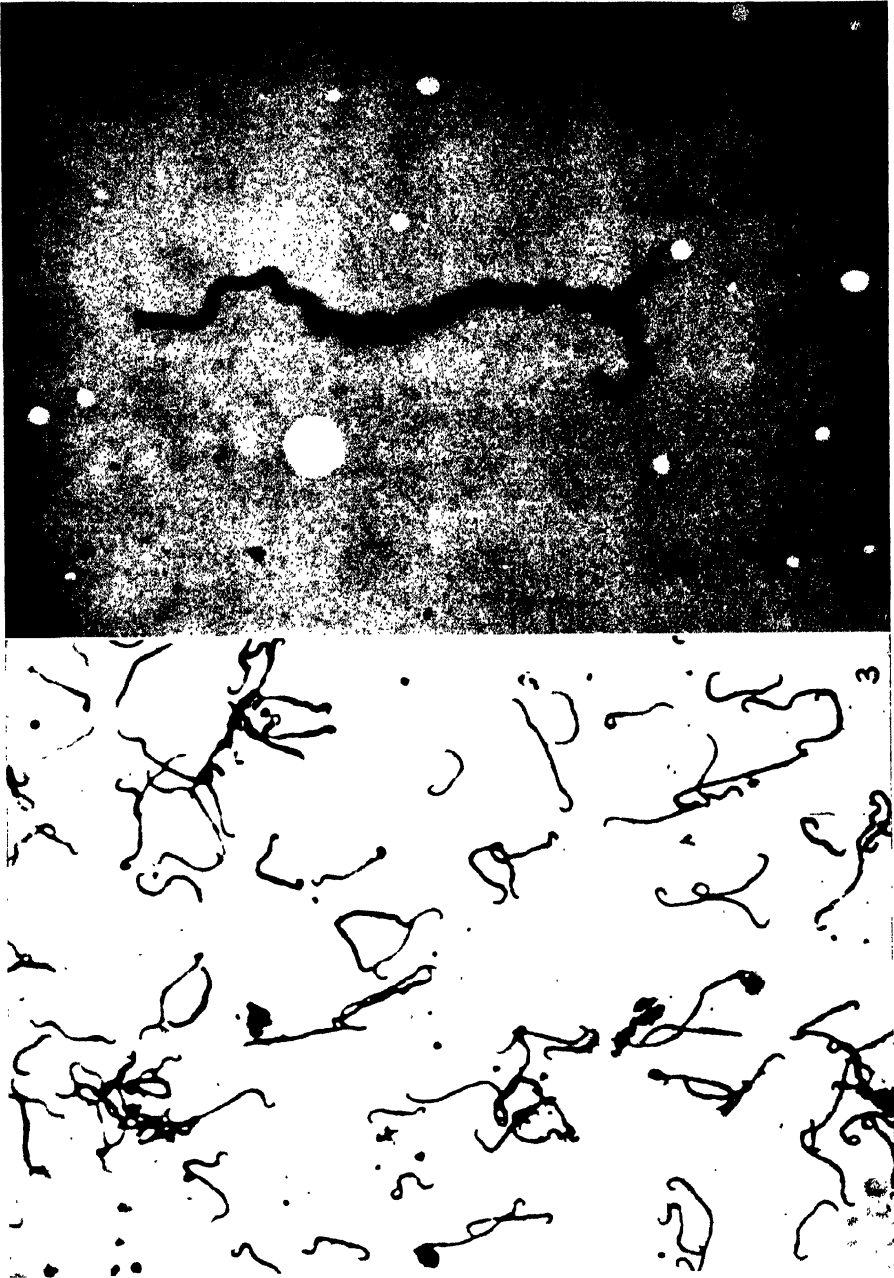


(Baker and Little: Leptospirosis in cattle)

PLATE 11

FIG. 3. Preparation of spirochetes from culture stained by Morosow's method. $\times 2267$.

FIG. 4. Electron microphotograph of spirochete that shows usual unit of size $90\text{ m}\mu$ in diameter and $4\text{ m}\mu$ long. $\times 23,200$.

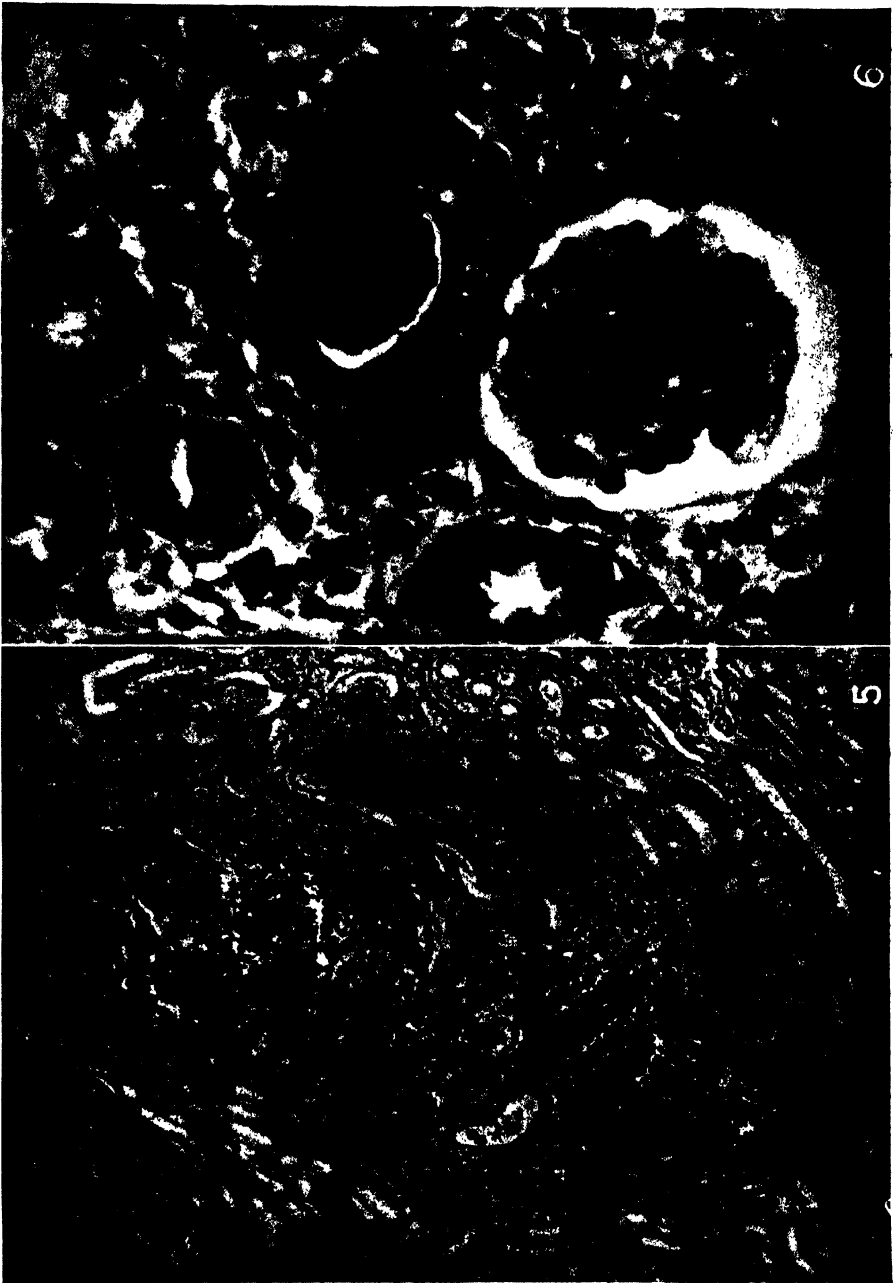


(Baker and Little: Leptospirosis in cattle)

PLATE 12

FIG. 5. Kidney from infected calf killed 28 days after inoculation. Stained with polychrome methylene blue. $\times 112$.

FIG. 6. Kidney from infected calf. Note cellular infiltration and tubular damage. Glomerulus appears normal. Stained with polychrome methylene blue. $\times 688$.



(Baker and Little: Leptospirosis in cattle)

RESISTANCE TO SPOTTED WILT IN TOMATO

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Spotted-wilt disease of the tomato (*Lycopersicon esculentum* Mill.) has been observed by the writer occasionally in past years in New Jersey and neighboring states. It has not appeared to be a very common disease nor has a locally severe outbreak been recognized until recently. The reason for the infrequency of its occurrence has not been obvious, for elsewhere in the world, notably in Australia (5), in New Zealand (2), and in the Union of South Africa (4), spotted wilt has proved disastrous in its effects on tomato cultivation.

The disease known as ringspot of dahlia (*Dahlia pinnata* Cav.), studied originally by Brierley (1), has yielded a virus similar to that from spotted wilt of tomato in greenhouse and laboratory tests. Infected plants of dahlia are a probable source of much of the tomato disease. The causative virus can overwinter in dahlia corms whereas it usually cannot overwinter in tomato, a short-lived perennial species that is grown in the New Jersey area commonly as an annual crop and to a much less extent as a winter crop in greenhouses. Ringspot of dahlia, like spotted wilt of tomatoes, has been a relatively unimportant disease in the past in the eastern United States.

An unexpectedly severe outbreak of ringspot was observed in an extensive planting of dahlias near Vineland, New Jersey, in 1946. In this planting, slightly more than 30 per cent of the plants were obviously affected by October 10. Probably additional plants were infected but were not readily recognized as diseased at the time. Tomato plants had failed that year, and for a few years preceding had been difficult to grow, in the immediate neighborhood.

A study was made of the affected dahlia plots with a view to learning why ringspot disease, which is believed to be spotted wilt as it occurs in dahlia, should be so common there and what factors may have influenced an unprecedented spread to tomatoes. A third plant was found to be involved conspicuously. Chickweed (*Stellaria media* L.) was very abundant throughout the dahlia planting. It showed many white or yellow circular spots on old leaves and some distorted young leaves, such as had not been seen elsewhere. From affected chickweed leaves, a virus comparable to that isolated from ringspot lesions in dahlia was obtained by subinoculation to plants of *Nicotiana glutinosa* L. and tomato. Moreover the disease on chickweed could be reproduced readily by mechanical inoculation of healthy chickweed plants with virus originally derived from ringspot lesions in dahlia. Despite the potentially

wide host range recognized for spotted-wilt virus (*Lethum australiense* H.), no other weed in the dahlia fields appeared to be affected. There were present, however, abundant thrips, presumably including the ubiquitous onion thrips, *Thrips tabaci* Lind. (Thripidae), a vector of spotted wilt.

The severity of the local outbreak of the disease can be accounted for largely on the basis of extensive dahlia culture during the months of June to October, in an area heavily infested with chickweed, a winter annual occurring most abundantly from September to July. The seasons for growth of dahlia and chickweed overlap and together encompass the entire year. Transfer of virus from infected chickweed would tend to increase the incidence of spotted-wilt lesions in dahlia each summer; return of virus to seedling chickweeds in the autumn would complete a cycle. Chickweed alone could not be expected to maintain the disease, because the weed does not persist through the summer season in any quantity; and, in fact, fields a few miles removed from dahlia culture had only healthy chickweed plants. By March of 1947 the chickweed in the dahlia-growing area had been destroyed by intensive cultivation except in a small plot. The plants in this plot were so numerous as to touch each other over most of the surface of the ground. Almost all were diseased. Shortly afterward these also were destroyed with a view to attempting control of the disease. Had they been left to grow until July, much renewed spread of disease to dahlia might have been anticipated.

Varieties of tomato resistant to spotted wilt in the Territory of Hawaii had become available recently through the work of Kikuta, Hendrix, and Frazier (3), who developed the Pearl Harbor tomato and several improved derivatives of this variety. The writer had observed the adequacy of resistance of the Pearl Harbor tomato in Honolulu. It seemed possible that the outbreak of disease in New Jersey might be controlled, so far as tomatoes were concerned, by the use of such resistant lines. The Hawaiian investigators kindly supplied suitable seeds for this experiment.

Unfortunately, it was found that the New Jersey disease overcame the type of resistance that is characteristic of the Pearl Harbor tomato, both under conditions of natural spread in the field and under experimental conditions in the greenhouse. In the field a partial but insufficient resistance was displayed (See table 1). In the greenhouse, where infection was more nearly simultaneous and hence involved on the average younger plants and where dosage of virus was probably greater, hardly a trace of resistance was noted in the Pearl Harbor tomato and its derivatives. All inoculations in the greenhouse were made by mechanical means, that is, by rubbing leaflets in the presence of freshly expressed extracts of 10 to 12-day-diseased tomato plants and fine (320-mesh) carborundum powder.

Fortunately, seeds of a large number of unusual varieties of tomato were available in the writer's seed collection at the time; many were grown and

seedlings produced from them were tested by mechanical inoculation. The virus used to test them was isolated originally from the dahlia by subinoculation to *Nicotiana glauca* and subsequent transfer from the characteristic necrotic-type lesions on this plant to seedlings of Bonny Best tomato, a fully susceptible variety. In tomato, it produced tip blight symptoms and other manifestations characteristic of the tomato malady in the field. In expressed juice this virus was completely inactivated in 10 minutes at 45°C. To maintain the virus at sufficiently high titer for use in testing seedlings, subinoculations were made at approximately 10-day intervals to new young tomato plants. Tests of the virus when thus regularly transferred showed sufficiently high infectivity to produce, in one test, an average of 42.8 lesions per leaf in 10 leaves of *N. glauca*.

Most of the varieties tested proved highly susceptible but two outstandingly resistant lines of tomato were found.

TABLE 1
Comparison of Tomato Varieties in the Field at Vineland, N. J., on July 15, 1947

Variety	Number of plants			
	Dead	Severely diseased	Moderately affected	Apparently healthy
Rutgers	26	10	4 ^a	6 ^a
Rey de los Tempranos	0	0	7	83
Pearl Harbor	17	40	6	27

^a Only plants in the last two columns, moderately affected and apparently healthy, had produced appreciable amounts of fruit.

One of the resistant tomatoes was known as Rey de los Tempranos (King of the Earlys). It was an Argentinian variety originally obtained through the kindness of Dr. M. F. Babb, of the Cheyenne Horticultural Field Station in Cheyenne, Wyoming. All plants of this variety seemed to be resistant (See figure 1). Many of them showed no obvious response to inoculation, though a little virus could be recovered sometimes from inoculated leaflets, indicating localized and inapparent infection. A few became diseased systemically, but later sent out healthy branches and produced fruits. Seeds from a fruit on such an injured plant were grown. A progeny of 56 plants produced from them seemed as resistant as the variety itself, only two being moderately affected, whereas all of 44 plants of the susceptible variety Bounty, grown as controls, became systemically diseased. In the field, resistance proved to be of a similarly high order; a few plants became affected but the planting as a whole appeared vigorous and fruitful, whereas neighboring plantings of the varieties Rutgers and Pan America were so severely affected as to produce no usable fruits.

The second variety of value was the Manzana (Apple) tomato. This was another Argentinian variety, received through the kindness of Dr. F. Rosenbusch of Buenos Aires. In this stock, the seeds of which were 10 years old when tested, some inhomogeneity existed. Part of the plants proved susceptible, but progenies that seemed wholly resistant were grown from seeds of resistant individuals.



FIG. 1. An unaffected tomato plant of the resistant variety Rey de los Tempranos and a severely injured plant of the susceptible variety Rutgers, each 17 days after inoculation with spotted-wilt virus originally isolated from ringspot of dahlia in New Jersey. (Photograph by J. A. Carlile.)

First-generation hybrids between the susceptible Rutgers tomato and the resistant Rey de los Tempranos were not so resistant as the variety Rey de los Tempranos nor so readily infected systemically as plants of the seed parent, Rutgers. The same was true of a similar cross having Bounty as susceptible parent, instead of Rutgers, and of the reciprocal hybrid between Rey de los Tempranos, used as female parent, and Bonny Best, a fully susceptible sort, used as male parent. In all cases the F_1 plants seemed intermediate between the parental types in their responses to inoculation.

In a second generation of the Rutgers cross, grown from seed produced by self-pollination of flowers on the F_1 plants above mentioned, typical spotted-

wilt disease was established in 299 plants of 401 inoculated; the remaining 102 plants reacted like the resistant variety Rey de los Tempranos, only 18 becoming somewhat affected later and these tending to send out healthy branches and to produce fruits. All of 397 inoculated control plants of the susceptible variety Rutgers became systematically diseased. The ratio of typically diseased to resistant plants among the second generation hybrids, 299:102, suggested that segregation for resistance had occurred in approximately a 3:1 ratio. A single-gene difference thus seemed to account for much if not all of the essential distinction between the resistant and susceptible stocks used in this test. The gene for resistance may be treated as though it were fully recessive in breeding experiments, although F_1 heterozygotes were shown to be more difficult to infect systemically than were the susceptible parent plants.

The Manzana tomato, as represented by selected resistant lines, was found to be as resistant as the variety Rey de los Tempranos in subsequent greenhouse and field tests in New Jersey. Manzana fruits were nearly spherical and of large size; they were red in color and of good flavor. Their only obvious defect was a tendency to concentric cracking and somewhat delayed ripening near the stem end of each fruit. Fruits of the variety Rey de los Tempranos were far more numerous and earlier in ripening, but were relatively small, flattened, and ridged.

The high degree of resistance of the variety Rey de los Tempranos in the field, the extreme susceptibility of the Rutgers tomato, and the intermediate but insufficient resistance of the Pearl Harbor tomato under New Jersey conditions are well shown in table 1.

Through the kindness of Mr. K. Kikuta and Dr. W. A. Frazier of the Agricultural Experiment Station at the University of Hawaii in Honolulu, Territory of Hawaii, the varieties found resistant in the field under New Jersey conditions were exposed to natural infection in Honolulu, on the grounds of the Agricultural Experiment Station, where a special test plot has been maintained for a study of resistance to Hawaiian spotted-wilt virus. Both Rey de los Tempranos and Manzana tomatoes appeared somewhat less resistant to spotted wilt there than the locally adapted resistant variety Pearl Harbor but much more resistant than the susceptible varieties Rutgers and Bounty. If the results of the preliminary test should be confirmed, this might indicate that the two types of resistance could be combined to advantage by crossing Pearl Harbor with Rey de los Tempranos or Manzana tomatoes. Among segregating progenies, some lines capable of resisting an increased variety of strains of spotted wilt might be found.

DISCUSSION

Spotted wilt is a viral disease of world-wide distribution. It has reached every continent and many oceanic islands. Infection seems to be much more

frequent in some areas than in others, probably in accordance with availability of suitable plant hosts to act as reservoirs and thrips to act as vectors of the causative virus. Measures for control of the disease have sometimes been based on isolation of crops from cultivated or weed hosts and sometimes on destruction of insect vectors. Until recently no success has been reported in the search for immune or highly resistant varieties.

The first adequate resistance in tomato to damage by locally-occurring strains of the virus was reported in 1945 by the Hawaiian group of investigators, Kikuta, Hendrix, and Frazier (3). These investigators introduced the Pearl Harbor tomato and several excellent derivatives, which are capable of withstanding the disease as it occurs in the Hawaiian Islands. The existence elsewhere of one or more additional strains of virus requiring another type of resistance in tomato is illustrated in the present investigation. Perhaps the virus will continue to disclose potentially dangerous strains, for which the now known types of resistance may be inadequate. Further selection of types of resistance may be needed to complete eventually the solution of the world-wide problem of control of spotted wilt in tomato.

Comparison of experiences in the Hawaiian Islands with subsequent studies in New Jersey teaches a somewhat unexpected lesson. Investigators in the Hawaiian Islands might logically have concluded that reintroduction of spotted-wilt virus would be without effect, because the Islands long have been infested by strains of spotted-wilt virus and in recent years have possessed also an adequate resistance in tomatoes. If spotted-wilt virus from New Jersey had been introduced, however, the adequacy of existing resistance in tomatoes would have been severely tested and perhaps nullified, unless the damaging new strains had found conditions there unsuited to their multiplication or transmission. Fortunately, adequate resistance for New Jersey strains of virus is now available if needed. The insight provided by tests of the Pearl Harbor type of resistance in New Jersey would suggest, however, that it is not advisable to introduce a virus into a country, especially one normally protected by ocean barriers, even though strains of the virus are reported as present already.

In general it has been supposed that a virus strain is capable of giving rise to all possible derivatives wherever it exists. There may, however, be practical limitations to selection and establishment of some variants. If mutation of one strain gives rise to a derivative especially well suited to potential host plants in an environment, the derived strain may persist and even become dominant. This evolutionary process may go on through a series of steps. Conceivably it may also reverse itself, so far as mutation is concerned, but suitable hosts to support the whole chain of events required for a gross change in characteristics or for a stepwise return may not be available, especially in a limited environment, such as that of an island or a small group of islands. For this reason certain strains may not be developed, selected, and established locally.

It will be of interest to test the two now known types of resistance in all the areas of the world from which spotted-wilt disease has been reported. Will the first-described virus of spotted-wilt, that reported from Australia by Samuel, Bald, and Pittman (5), resemble that of the Hawaiian Islands, that of New Jersey, or neither of these, in its effect on tomato plants bearing the Pearl Harbor type and on those bearing the newly described type of resistance? Will the so-called kromnek disease in the Union of South Africa (4) resemble spotted-wilt disease as first recognized in Australia in its relationship to the two types of resistance? Whereas it would be obviously unsafe to import spotted-wilt viruses from different continents for comparison with one another, it would seem to be a safe and expedient process to send seeds of resistant tomatoes to all affected areas.

SUMMARY

An outbreak of spotted-wilt disease in a small area of southern New Jersey was caused by a strain or strains of the causative virus capable of overcoming the resistance of the Pearl Harbor tomato and its recent derivatives as developed in the Hawaiian Islands. A heritable resistance adequate for control of the New Jersey disease was found in two kinds of South American tomato, the variety Rey de los Tempranos and some lines of the variety Manzana, both from Argentina. The new type of resistance, as exemplified in the variety Rey de los Tempranos, was inherited as a single Mendelian factor in crosses with the susceptible Rutgers variety of tomato; approximately one quarter of all plants (102 of 401) in the second hybrid generation displayed resistance comparable to that of the resistant parent.

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DETERMINATION OF SEDIMENTATION CONSTANTS IN THE SHARPLES SUPERCENTRIFUGE

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INTRODUCTION

The high-speed centrifuge in recent years has become an invaluable tool in many branches of colloid and biochemical research. In addition to the technical improvements enabling the attainment of centrifugal fields as high as 500,000 times gravity, there have been developments in the optical methods used for study of the sedimenting material. The brilliant researches of The Svedberg and his collaborators (15) have culminated in the development of the ultracentrifuge. Centrifuges of another design have been developed by Beams and coworkers (2), Bauer and Pickels (1), and Wyckoff and Lagsdin (17) with the logical addition of a quantity-type rotor for centrifuging moderate quantities of liquids. The work of Stanley (12) has shown this quantity-type centrifuge to be of inestimable value in the isolation and purification of viruses. However, the need for a less expensive centrifuge capable of continuous flow operation and of sedimenting particles as small as the viruses resulted in investigations concerning the possibilities of the Sharples supercentrifuge.

Hauser *et al.* (4, 5) devised an efficient procedure for fractionating bentonite suspensions into several reasonably monodisperse fractions by utilizing the theory and equations derived for the Sharples supercentrifuge. It was also demonstrated (5) that, with very little effort, the size of a well-defined fraction of particles could be evaluated. Success in quite a different field was experienced by Stanley (13, 14), who sought a better method for the isolation of viruses on a large scale. In view of the practical value of the Sharples supercentrifuge in the purification of tobacco mosaic and influenza viruses, it seemed worthwhile to reexamine the theory of sedimentation in the supercentrifuge in an attempt to adapt it for the calculation of the sedimentation constants of those materials capable of being sedimented in fields of about 60,000 times gravity. An estimate of the reliability of the supercentrifuge method could then be made by comparing the results with those obtained by direct measurement in the ultracentrifuge.

THEORETICAL

Hauser and Reed (4) assumed that every particle in the bowl of the Sharples centrifuge is subjected to two velocity components, one perpendicular and the other parallel to the axis of rotation of the bowl. The first, whose magnitude is dependent on the centrifugal field, can be expressed quantitatively by a modified Stokes law for falling bodies; the second is proportional to the rate of flow of

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material through the bowl and can be defined by Newton's law of viscous flow. Under given experimental conditions the point at which a particle hits the wall will be a function of the point of departure and the effective mass and shape of the particle. This can be expressed by

$$Y = F(X_0, m_e, f) \quad (1)$$

where Y is the distance in centimeters from the top of the straightening vanes to the point at which the particle settles, m_e is the effective mass of the particle, f is the frictional coefficient of the particle, and X_0 is the distance from the axis of rotation at which sedimentation started (figure 1). The velocity component in the x direction can be expressed by

$$\frac{dx}{dt} = \frac{m_e \omega^2 x}{f} \quad (2)$$

where ω is the angular velocity of the centrifuge bowl in radians per second. Reed (9), following the treatment of Lamb (6), found

$$\frac{dy}{dt} = \frac{Q_{\min.} K}{60\pi(R_2^2 - R_1^2)} \left[R_1^2 \ln \frac{x}{R_2} + \frac{R_2^2 - x^2}{2} \right] \quad (3)$$

for the velocity component parallel to the axis of rotation.² In the present work the bowl has the dimensions $R_1 = 0.734$ cm., $R_2 = 2.22$ cm., and $K = 1.11$, and $Q_{\min.}$ is the rate of flow of solution through the bowl in milliliters per minute. Combining equations 2 and 3 and integrating between the limits $x = X_0$, $y = 0$ and $x = R_2$, $y = Y$ leads to equation 4:

$$Y = \frac{Q_{\min.}}{60\pi(R_2^2 - R_1^2)} \cdot \frac{f}{m_e} \cdot \frac{K}{\omega^2} \left[\frac{R_2^2}{2} \ln \frac{R_2}{X_0} - \frac{R_1^2}{2} \left(\ln \frac{R_2}{X_0} \right)^2 + \frac{X_0^2 - R_2^2}{4} \right] \quad (4)$$

² Lamb (6) derived the equation

$$\frac{dy}{dt} = -\frac{p_1 - p_2}{4\eta l} x^2 + A \log x + B \quad (3a)$$

for the velocity of flow of liquid in a pipe of uniform circular section where l is the length of the section under consideration, $(p_1 - p_2)$ is the pressure drop across that section, η is the viscosity of the medium, and A and B are constants of integration. In the case of flow in the centrifuge bowl the treatment differs from that of Lamb in that the boundary conditions for integration are those of a concentric shell of fluid rather than a solid tube of fluid. The constants A and B are evaluated by the use of the boundary conditions, $dy/dt = 0$ at $x = R_2$, and $\frac{d}{dx} \left(\frac{dy}{dt} \right) = 0$ at $x = R_1$. Combination of the resulting equation with the equation for the flux across any section,

$$Q = \int_{R_1}^{R_2} \frac{dy}{dt} 2\pi x \, dx$$

leads to equation 3.

Following the notation of Svedberg (15), the sedimentation constant, s , is defined as

$$s = \frac{1}{\omega^2 x} \cdot \frac{dx}{dt}$$

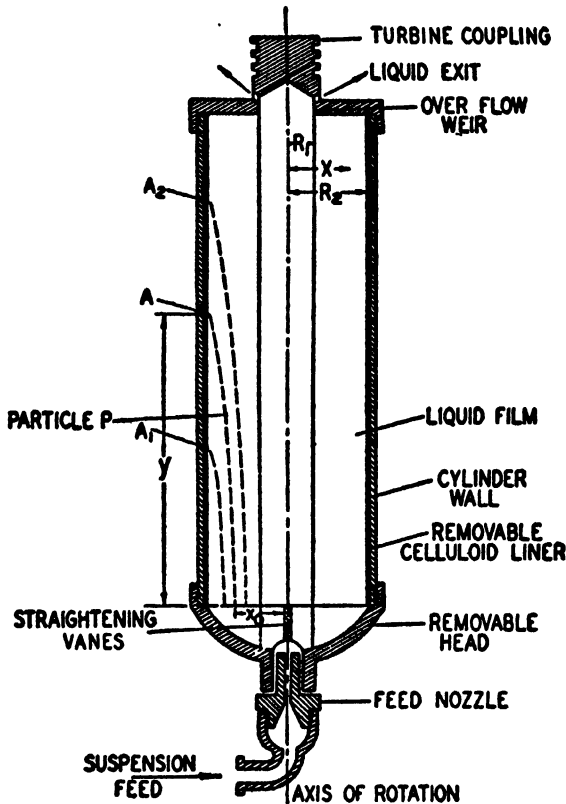


Fig. 1. Section through supercentrifuge bowl

which, according to equation 2, is equal to m_o/f . Substituting for the appropriate constants, correcting radians per second to R.P.M., and rearranging terms leads to equation 5

$$s = \frac{0.1097 Q_{\min.} \cdot C}{(\text{R.P.M.})^2 \cdot \bar{Y}} \quad (5)$$

where $C = 0.6235 + 0.277 X_0^2 - 5.19 \log X_0 - 1.58 (\log X_0)^2$.

According to the treatment proposed by Hauser *et al.* (4, 5) it is expected that under a given set of experimental conditions the larger particles in the solution would settle out on the liner sooner than the smaller particles. Still smaller

particles would pass out of the bowl, because insufficient time is allowed for the centrifugal force to act. If the rate of input of solution is decreased or the centrifugal force increased, some of the smaller particles would also settle onto the wall of the centrifuge bowl. It thus would be possible to vary the yield of sedimentation and the distribution of particles along the centrifuge bowl by changing the experimental conditions. At very low rates of flow most of the particles would be found at the bottom of the bowl and a good yield would result. At higher rates of flow the distribution of sediment along the bowl would be approximately uniform but the yield poor, because many particles would pass out the overflow weir. It follows that there would exist an optimum set of operating conditions combining high yield and large capacity. In general, the distribution of particles along the bowl after a run under optimum conditions would be non-uniform, with more particles at the bottom than at the top. In the special case in which the solution under investigation contains particles covering but a small range of size and shape, the distribution of sediment obtained under optimum operating conditions would approach uniformity with only slightly more particles at the bottom of the bowl than at the top. This is to be contrasted with the case of solutions containing particles of many sizes. Under such circumstances the distribution of sediment obtained under optimum conditions would not approach uniformity. The rate of flow which gives a nearly uniform layer would be too fast for the settling of the small particles in the suspension and a poor yield would result. Similarly, at a rate of input slow enough to produce a good yield, the large particles would settle at the bottom of the bowl and the resulting layer would be decidedly non-uniform.

Equation 5 applies to solutions containing any distribution of particle sizes, and it can be simplified for the special case of solutions of homogeneous material. When such solutions are used there will be but one particle size at each point on the bowl; conversely, a particle at a given value of Y must have started from a unique value of X_0 . It can thus be assumed that $Y = f_1(X_0)$. Figure 2, an empirical chart illustrating $Y = f_1(X_0)$, was obtained by means of a careful study of a fractionated bentonite suspension which was analyzed for particle size by measuring experimentally the weight distribution along the bowl and calculating the particle size distribution by the equations derived by Hauser and Reed (4). The calculations indicated that the values of X_0 for a definite value of Y did not vary much for different particle sizes. This, of course, is true only if the size distribution curve is sharp and the sedimentation along the bowl is nearly uniform and in good yield. In this way, average values of X_0 for the different Y values were obtained empirically. Since C is a function of X_0 , it is possible to obtain $C = f_2(Y)$. In view of the approximations involved in this treatment an average sedimentation constant can be calculated by using the value $C = 0.32$ for $Y = 10$ (10). Substituting for C and Y into equation 5 leads to:

$$s = 0.0035 \frac{Q_{\min.}}{(\text{R.P.M.})^2} \quad (6)$$

It must be restated that equation 6 can be used for the calculation of sedimenta-

tion constants only if the experimental conditions are optimal so that an approximately uniform layer of sediment and good yield are obtained.

EXPERIMENTAL

In a detailed series of experiments, Stanley (13) found that yields of tobacco mosaic virus as high as 93 per cent were obtained by passing the virus solution through the laboratory-model Sharples supercentrifuge operating at 50,000 R.P.M. with the rate of input of solution about 15 ml. per minute. Using equation 6 and these data a value of 210 S for s is obtained. To test the sensitivity and

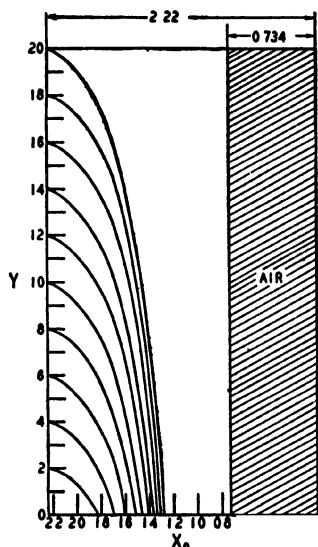


FIG. 2. Paths of the particles in the bowl

accuracy of the method, further experiments were conducted with tobacco mosaic virus.

A stock solution containing 60.2 mg. of tobacco mosaic virus per milliliter, which had been partially purified by precipitation with ammonium sulfate, was used. Three liters of a solution containing 3.23 mg. of protein per milliliter in distilled water were fed into the centrifuge bowl at 12.1 ml. per minute at a temperature of 21°C. The angular rotation of the bowl was 50,000 R.P.M. Upon inspection, approximate uniformity of sedimentation of the virus along the entire length of the liner was observed with a yield of about 91 per cent. Substitution of the appropriate quantities into equation 6 leads to a value of 170 S for the sedimentation constant of tobacco mosaic virus under the conditions of the run. A certain amount of ambiguity necessarily complicates a complete understanding of the true sedimentation rate, owing to the temperature gradients that were

present. It was found that the liquid which drained from the bowl on stopping was at a temperature of about 26°C., despite attempts at cooling by incorporating into the otherwise regular supercentrifuge a cooling coil, through which water at about 0°C. was passed. The temperature of the effluent unfortunately cannot be used to advantage because of the evaporation and accompanying cooling of the liquid as it leaves the bowl and passes through the exit spout through which air is constantly circulating. Until more accurate determinations of temperature conditions in the bowl itself are made, the value obtained from the drainings will be used. Correcting the value of s at 26°C. to the reference temperature of 20°C. yields 147 S (uncorrected for viscosity and density of the medium because of lack of knowledge of impurities). The value 159 S (also uncorrected for the medium) was obtained by a study in the analytical ultracentrifuge of the Bauer and Pickels type equipped with the Svensson optical system. The agreement of the value obtained with the Sharples supercentrifuge with that obtained with the reliable ultracentrifuge was good, for the discrepancy was only about 8 per cent.

In the second experiment two batches of virus solution were prepared containing 2.91 mg. of protein per milliliter. One solution was run through the machine at 14 ml. per minute at a temperature of 27°C. with the resultant yield of 84.5 per cent. The second solution was passed through the bowl at 12.5 ml. per minute. In this case the yield was 85.4 per cent, a value not significantly different from the previous run. More efficient cooling was obtained during the second run, with the result that the temperature was very nearly constant at 22.5°C. Values of s_{20} equal to 166 S and 165 S were obtained in these runs. The analytical ultracentrifuge gave a value of 169 S for s_{20} , in excellent agreement with the values obtained by the Sharples supercentrifuge.

A third experiment was conducted to determine the uniformity of the sediment along the centrifuge bowl. About 1600 ml. of a solution of purified tobacco mosaic virus in distilled water containing 3.06 mg. of protein per milliliter was passed through the centrifuge, rotating at 50,000 R.P.M., at 11.4 ml. per minute. The use of a brine-ice mixture to cool the centrifuge bowl served to reduce the operating temperature so that the bowl drainings were at 14.5°C. The yield in this run was 78 per cent and the plot of cumulative weight per cent *vs.* distance in centimeters along the bowl was approximately linear. A value of s_{20} of 183 S was calculated for this run by means of equation 6. The value $s_{20} = 170$ S was obtained by a study of the same material in the ultracentrifuge.

DISCUSSION

Under controlled experimental conditions, as shown in the above experiments, the error in evaluating the sedimentation constant of tobacco mosaic virus by means of the Sharples supercentrifuge is small. In order that equation 6 be satisfied, the material under investigation must be in a reasonably homogeneous state, as in the cases of purified viruses or fractionated bentonite. Another precaution to be observed concerns the rate of input of solution. Optimum oper-

ating conditions must be adhered to, so that the material will sediment in yields of about 85 per cent and with approximate uniformity of sediment along the bowl. Obviously the supercentrifuge is incapable of efficiently sedimenting materials as small as egg albumin, but it is useful for materials, like the viruses, with sedimentation constants of about 100 S and larger.

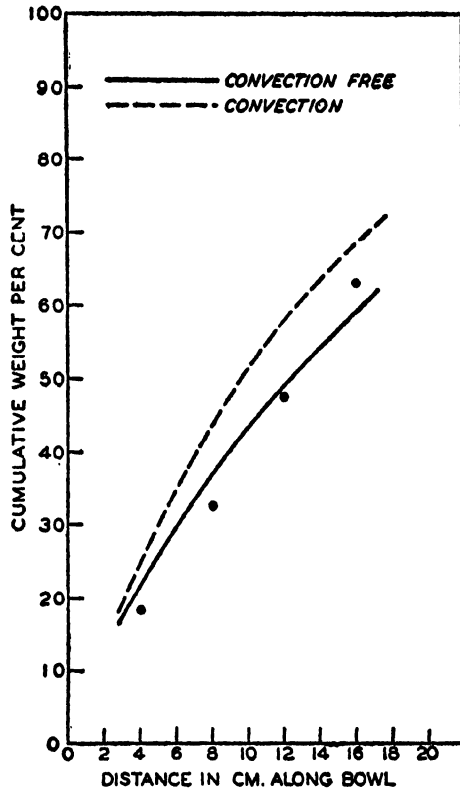


FIG. 3. Plot of calculated cumulative weight per cent vs. distance in centimeters along the bowl. ● represents experimental data.

Of interest is the fact that the data of figure 3, corresponding to the third experiment, can be used to calculate the sedimentation constant of the virus without assumptions regarding $Y = f_1(X_0)$ and without the empirical calibration factor, $C = 0.32$ at $Y = 10$.

Hauser and Reed (4) showed that the number of particles which have settled out on the wall before point A (figure 1) is reached is proportional to the ratio of the total volume of solution flowing across the area enclosed by the concentric circles of radii X_0 and R_2 to the total amount of suspension fed into the bowl. Since the weight of the particles is proportional to their number this relationship

can be expressed as

$$W_r = W \frac{\int_{x_0}^{x_1} \frac{dy}{dt} 2\pi x dx}{Q} \quad (7)$$

where W_r is the weight of particles which have settled out at a given point and W is the total weight of particles entering the bowl. The values of the cumulative weight per cent, $(W_r/W) \times 100$, are determined experimentally. Equation 3 gives dy/dt as a function of x , and thus the integral of equation 7 is in terms of X_0 alone. However, X_0 depends on S and Y in a complicated fashion, thereby presenting almost insuperable obstacles to an exact solution of equation 7. By a series of approximations it is possible to solve the equation. A table for each assumed value of S is set up containing the values of Y considered, the values of C calculated from equation 5 for the known experimental conditions, the values of X_0 equivalent to the calculated C , and finally a column corresponding to

$$\frac{1}{Q} \int_{x_0}^{x_1} \frac{dy}{dt} 2\pi x dx$$

which is equal to the theoretically determined cumulative weight per cent. For the purposes of comparison a column is included which tabulates the experimental values of the cumulative weight per cent. The integral in equation 7 has been evaluated for the centrifuge bowl involved in these studies and can be expressed by equation 8 (10):

$$\frac{1}{Q} \int_{x_0}^{x_1} \frac{dy}{dt} 2\pi x dx = 1.2 - 0.3125X_0^2 \log X_0 - 0.445X_0^2 + 0.632X_0^4 \quad (8)$$

Table 1 represents a sample calculation for the assumed value $s = 160 S$.

This calculation is repeated for several assumed values of the sedimentation constant and the assumed value which gives the best correlation between the experimental and theoretical values for the cumulative weight per cent is considered to be the most nearly correct. Table 2 shows the results of the calculations for several different assumed sedimentation constants. These results indicate clearly that $s = 130 S$ and $s = 190 S$ cannot be considered as solutions of equation 8. Similarly, it is clear from the results presented in the table that values corresponding to $s = 150 S$ to $s = 170 S$ represent the best solution. The table also shows that no single value of the sedimentation constant can completely account for the distribution of the virus sedimented on the walls of the centrifuge bowl. This is probably due to the limitations of the theory proposed in this treatment. Under the conditions of operation of the centrifuge it is likely that streamline flow is not realized completely and the end effects at the bottom and top of the bowl are not negligible, as was assumed in the theory. It is of interest that the value obtained by the approximate treatment as expressed by equation 6 is in very good agreement with the value obtained by the more tedious and complicated method involving the cumulative weight distribution curve.

Of additional interest is the fact that the calibration factor used in obtaining equation 6 was obtained from studies on a fractionated bentonite suspension and applied with considerable accuracy to the experiments on tobacco mosaic virus.

The studies conducted by Stanley on the sedimentation of influenza virus in the Sharples supercentrifuge prove of interest in a further evaluation of the theory presented for the sedimentation in the centrifuge. Stanley (14) concluded that the infectious allantoic fluid can be passed through the centrifuge at rates of flow between 40 and 50 ml. per minute with the recovery of approximately 80 per cent or more of the biological activity. His studies were conducted at 50,000 R.P.M. According to equation 6 the sedimentation constant of the influenza

TABLE 1
Sample calculation for the assumed value $s = 160 S$
 $s = 160 S$; $s_{20} = 184 S$

Y	C	X_0	$[(W_T/W) \times 100]$ (THEORY)	$[(W_T/W) \times 100]$ (EXPERIMENT)
4	0.128	1.72	22	18
8	0.256	1.53	37	33
12	0.384	1.39	49	47
16	0.512	1.271	59	63

TABLE 2
Results of calculations for several assumed sedimentation constants

Y	$[(W_T/W) \times 100]$ (THEORY)					$[(W_T/W) \times 100]$ (EXPERIMENT)
	$s = 130 S$ $s_{20} = 149 S$	$s = 150 S$ $s_{20} = 172 S$	$s = 160 S$ $s_{20} = 184 S$	$s = 170 S$ $s_{20} = 195 S$	$s = 190 S$ $s_{20} = 218 S$	
4	18	21	22	23	25	18
8	32	36	37	39	42	33
12	43	47	49	51	55	47
16	52	57	59	62	67	63

viruses would be about 630 S. The most frequently reported values of the sedimentation constants of the strains of influenza virus lie between 600 and 700 S. Again, the results with the Sharples supercentrifuge are in excellent agreement with the value obtained by the reliable ultracentrifuge technique.

It is of interest that Markham (8), following the approach of Bechhold and Schlesinger (3) and of Schlesinger (11), calculated the sedimentation constant of tobacco mosaic virus on the basis of completely stirred sedimentation. Making use of the formula for convective centrifuging,

$$\frac{C_t}{C_0} = e^{-(R_2 \omega^2 s \Delta t / r)} \quad (9)$$

he calculated values for the sedimentation constant which were in satisfactory

agreement with those found by Lauffer (7) by means of the ultracentrifuge. In equation 9, C_t is the concentration of virus in the supernatant fluid at time t , C_0 is the initial concentration of virus, A is the area of the internal surface of the bowl (279 cm.²), and V is the volume of solution that has passed through the bowl in the time t . As Markham pointed out, equation 9 applies to the most unfavorable conditions for sedimentation, and the sedimentation constant values derived from it should be minimal. That they were, in fact, larger than the accepted value for tobacco mosaic virus could mean, as Markham indicated, that there was some concentration gradient formation in the supernatant. In that case sedimentation would occur more rapidly than expected and the values obtained by equation 9 should be high. Table 3 shows the results obtained from the present data for the sedimentation constants on the basis of convectionless sedimentation as expressed by equation 6 and under the conditions of convective centrifuging calculated by equation 9. In all experiments except the last the values obtained on the basis of stirred sedimentation are too high. The value, 840 S, for the sedimentation constant of influenza virus is obtained by the use of

TABLE 3

Comparison of experimental results with values calculated from two theories

Q _{min.}	YIELD	SEDIMENTATION CONSTANTS, S		
		Equation 6	Equation 9	Ultracentrifuge
<i>ml./min.</i>	<i>per cent</i>			
12.1	91	147	248	159
14	84.5	166	217	169
12.5	85.4	165	222	169
11.4	77	183	188	170

the data of Stanley (14) and equation 9. As in the case of most of the experiments with tobacco mosaic virus, the sedimentation constant for influenza virus calculated on the basis of convective centrifuging is too high.

In an attempt to evaluate further the two theories for sedimentation in the Sharples supercentrifuge the cumulative weight per cent curve was calculated in the following manner on the basis of convective centrifuging:

$$\frac{dC}{dt} = -\frac{R_2 \omega^2 s A}{V} C \quad (10)$$

and

$$\frac{dC}{dt} = \frac{dC}{dy} \cdot \frac{dy}{dt} \quad (11)$$

In equation 11, dy/dt is the velocity of flow through the bowl, which is $Q/\pi(R_2^2 - R_1^2)$. Substituting for dy/dt in equation 11, equating 10 and 11, and integrating the resulting equation leads to:

$$\frac{C_T}{C_0} = e^{-R_2 \omega^2 s A \pi (R_2^2 - R_1^2) T / V Q} \quad (12)$$

In equation 12, C_Y is the virus concentration of the supernatant at point Y , V is now the volume of liquid contained in the bowl, and the other terms are the same as defined earlier. By the use of equation 12 it is possible to calculate the per cent of virus sedimented at any point along the centrifuge bowl. Figure 3 shows the results obtained by the two methods along with the experimental points. It can be seen that the results based on convectionless sedimentation are in closer agreement with experiment.

Of some interest is the attempt at increasing the efficiency of the Sharples supercentrifuge by decreasing the volume of the bowl through an increase in R_1 (14, 16). Stanley (14), in studies on tobacco mosaic and influenza viruses, found that no significant increase in efficiency resulted from decreasing the liquid layer. Qualitatively, that is the result expected on the basis of convection-free sedimentation. Although the decrease in the distance, x , along which the particle sediments would tend to increase the efficiency, it is mainly compensated by the decrease in the time the particle is in the bowl. Favoring a slight increase in efficiency is the fact that the particles are subjected to higher average centrifugal fields because of the larger values of X_0 . The use of V in equation 9 as the total amount of fluid passing through the bowl implies a rapid flow of solution. Under such conditions no significant increase in efficiency would be expected on the basis of stirred sedimentation if the liquid layer were decreased. It is of considerable interest that a decrease in the liquid layer by an increase in R_1 would enable the sedimentation of particles which are too small to be centrifuged out in the conventional clarifier bowl. In this way the practical usefulness of the Sharples supercentrifuge could be greatly enlarged. A detailed analysis of the sediment along the bowl for different values of R_1 would be necessary before a quantitative evaluation of the resolving power of the centrifuge could be made.

Complete accord with experiment cannot be expected, because of the simplifying assumptions and approximations made in the theoretical treatment. The existence of a density gradient in the bowl in a direction perpendicular to the intense centrifugal field would lead to instability, with a resultant tendency for the sedimenting particles to be distributed uniformly along the length of the bowl. The fact that non-uniform layers of sediment are obtained under some experimental conditions indicates that this cannot be a dominant factor. However, a true picture of conditions in the bowl must necessarily include the contribution of convection caused by the density gradient along the bowl.

SUMMARY

An extension and modification of earlier theoretical work on the Sharples supercentrifuge was performed. With the aid of a calibration factor obtained by a detailed study on a fractionated bentonite suspension, the sedimentation constant of many substances can be calculated from a simple equation derived on the basis of convection-free sedimentation. Experiments with tobacco mosaic virus designed to test the sensitivity and accuracy of the method were described. Excellent agreement was obtained by a comparison of the calculated results with those obtained in the more elaborate ultracentrifuge. A theory for

sedimentation in the Sharples supercentrifuge on the basis of complete convection was discussed and the results obtained by its use were presented. An attempt at evaluating the relative contribution of convection was made by an analysis of the weight distribution of sediment along the wall of the centrifuge bowl. The results indicated that the equations based on convection-free sedimentation yield values which are in closer agreement with experimental values.

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STUDIES ON A NEW CORN VIRUS DISEASE

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INTRODUCTION

A corn virus disease not previously reported was found in California⁹ and Texas¹ during the summer of 1945. Diseased specimens received by the writer from both States* were examined and tentatively judged not to be affected by corn stripe,¹² a malady which the disease resembled superficially. The new infection, which was found to be caused by a virus of the yellows type, was given the name stunt.

Transmission of corn stunt virus by the leafhopper *Baldulus maidis* (De L. and W.) was reported in September 1946.¹⁶ In a later publication¹⁷ the virus was shown to require a long incubation period in its vector. Results of further studies will be recorded here.

SYMPTOMS

Symptoms of corn stunt were first described by *Frazier* in 1945.⁹ A little later in the same year they were described by *Alstall*.¹ The following year stunt was described by *Pickett et al.*¹⁸ Since all of the descriptions were brief and based entirely on field observations, the writer will describe the disease as it developed in pot-grown corn plants in greenhouses at Princeton, New Jersey.

The first symptoms usually appeared at the bases of newly developed leaves on one or both sides and consisted in small chlorotic spots, sometimes circular but often elongated. The spots varied in area from pin points to blotches of considerable dimensions. Early stages of the disease are shown in Figure 1. In successive leaves above that bearing the first symptoms the chlorotic areas extended further and further toward the tips until finally entire leaves were involved. Thus, leaves at the top of a plant become diseased throughout. No visible lesions appeared around leafhopperfeeding punctures and clearing of veins was not observed. There was a stage in the development of chlorosis

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in which affected tissues were confined to areas along veins. The veins affected usually were not the largest but were those midway between the largest veins. In this stage the disease produced typical striping patterns such as are shown in Figure 2. The chlorotic patterns in the first leaves to show disease were produced by chlorotic spots and stripes on a green background; the chlorotic patterns in later leaves by green spots and stripes on a chlorotic background. Leaves that developed after the virus became fully systemic were chlorotic throughout. In thus producing a generalized chlorosis stunt differed from all



FIG. 1. Bases of 3 leaves from diseased corn plants of the variety New Jersey hybrid No. 2, showing early stages of the chlorosis caused by stunt virus. Photographed by *J. A. Carlile*.

other known corn virus diseases. In Figure 3 a section of a leaf that was practically devoid of chlorophyll is presented beside a section of a comparable leaf from a healthy plant. There was a tendency for diseased leaves to become slightly tinged with red; also, there was a tendency for diseased stalks to become reddish. Chlorosis usually did not appear in stalks until a late stage of the disease was reached, and then only in the upper parts of stalks, in most cases. The upper internodes were much shortened but affected stalk tissues never collapsed and never showed necrotic lesions. Stunt caused the production of an abnormally large number of secondary shoots in the axils of leaves and along branches bearing ears. It also stimulated growth in the husks of ears

causing them to produce abnormally long tips. Thoroughly diseased plants produced very small ears. However, stunt did not prevent the development of normal-appearing viable seeds.

In its late stages the disease sometimes affected roots. Virus apparently was slow to move into roots but when it reached them it caused stunting and the production of innumerable branches. This feature was especially striking when brace roots became affected. The bases of two comparable plants of

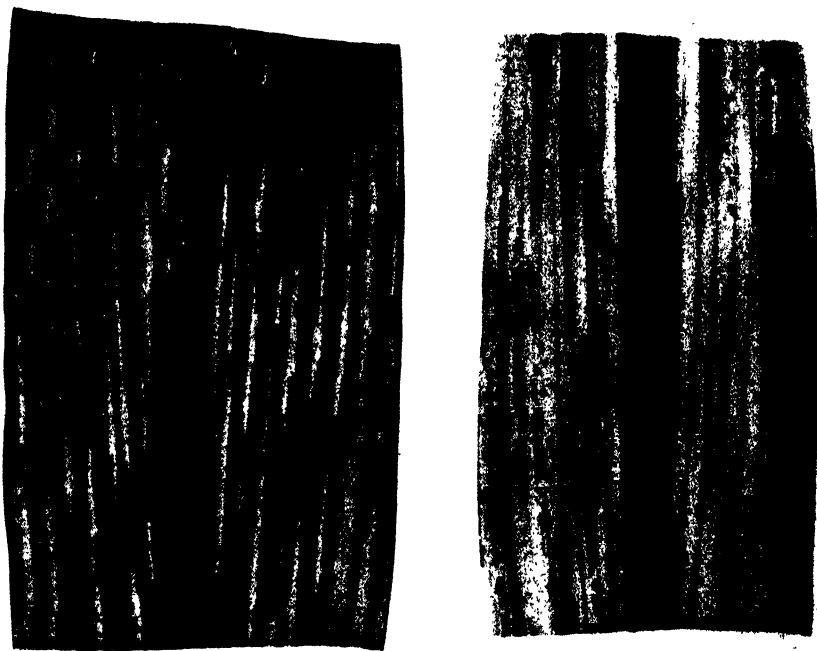


FIG. 2. Sections of leaves of diseased corn plants of the variety New Jersey hybrid No. 2, showing two distinct striping patterns. Photographed by J. A. Carlile.

the same age are presented in Figure 4. The roots of the diseased stump shown on the left are stunted and excessively branched; the roots of the healthy stump at the right are of normal size and show the normal amount of branching. Microscopic examinations of diseased leaf, stalk and root tissues failed to reveal the presence of inclusion bodies. In this respect stunt differed strikingly from stripe of corn¹² with which conspicuous inclusion bodies are associated.

Baldulus maidis

Baldulus maidis, the vector of stunt,¹⁶ is an active, slender, straw-colored leafhopper varying in length usually from $3\frac{1}{2}$ to $4\frac{1}{2}$ millimeters. It flourishes



FIG. 3. Sections of leaves from corn plants of the variety New Jersey hybrid No. 2. The section at the left shows a late stage of the disease; that at the right is from a healthy plant. Photographed by *J. A. Carlile*.

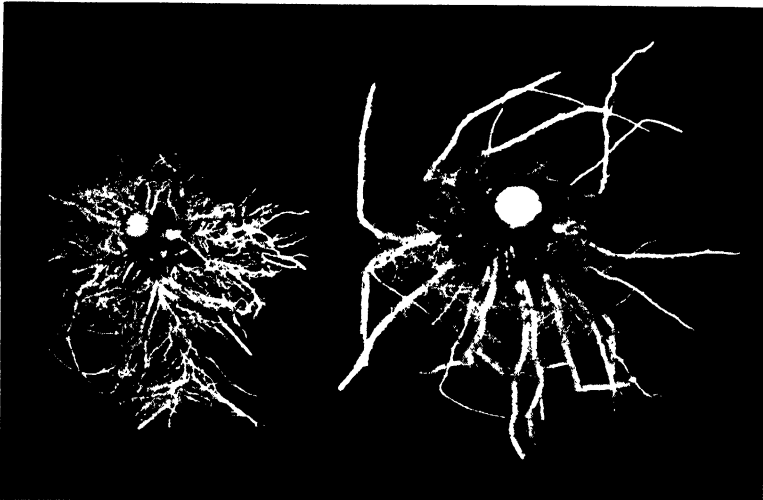


FIG. 4. Stumps of 2 corn plants of the variety New Jersey hybrid No. 2. The stump at the left is from a stunt plant and bears dwarfed, abnormally branched roots. The stump at the right is from a healthy plant with normal roots. Photographed by *J. A. Carlile*.

on corn and does well on teosinte. It does not thrive on any of the other plants that have been tested. The insect was cultured continuously during the past two years. Its development on corn was observed.

A large colony of egg-laying females was confined on a healthy corn plant from October 29 to October 31. Many eggs were deposited in the plant. Late in the afternoon of November 8 the plant was carefully observed; there were no nymphs. By ten o'clock the next morning a few of the eggs had hatched. One newly hatched nymph was placed on a short piece of freshly cut corn stalk in each of ten vials, the mouths of which were covered with cheesecloth. The pieces of corn stalk were renewed from time to time. The

TABLE 1
Development of Baldulus maidis in Cultures

Indi- viduals observed	Development		Lengths of time required by different instars
10	1st instar of nymphs hatched 5:00 P.M. 11/8 to 10:00 A.M. 11/9	shed skins 11/14	1st instar lasted about 5 days
10	2nd instar of nymphs hatched 5:00 P.M. 11/8 to 10:00 A.M. 11/9	" " 11/19	2nd instar last- ed 5 days
10	3rd instar of nymphs hatched 5:00 P.M. 11/8 to 10:00 A.M. 11/9	" " 11/21	3rd instar lasted 2 days
6	4th instar of nymphs hatched 5:00 P.M. 11/8 to 10:00 A.M. 11/9	" " 11/22	4th instar lasted 1-2 days
4	4th instar of nymphs hatched 5:00 P.M. 11/8 to 10:00 A.M. 11/9	" " 11/23	
10	5th instar of nymphs hatched 5:00 P.M. 11/8 to 10:00 A.M. 11/9	" " 11/27	5th instar lasted 4-5 days

temperature at which the vials were held varied from 72° to 75° F. The growing insects were observed twice each day, once about ten o'clock in the morning and once about four o'clock in the afternoon. The results of the observations are summarized in Table 1. It will be seen that 18 days were required for newly hatched nymphs to reach the adult stage under conditions of this test. A few of the eggs hatched early on the 12th day after they were deposited. At somewhat higher temperatures eggs were hatched on the 11th day after deposit. Development from egg to adult required about 30 days.

Efficiency of Baldulus maidis in Transmission of Stunt

Soon after *Baldulus maidis* had been found to transmit stunt, experiments were undertaken to determine its efficiency as a vector. In one of the experiments 2 colonies of 50 adults each, consisting of insects that had been reared on stunt corn plants, were transferred at daily intervals for a period of 24

days to healthy young corn plants growing in 4-inch pots. There were from 2 to 4 plants in each pot. At the same time 2 colonies, consisting of 50 virus-free adults each, were transferred at daily intervals to similar healthy corn plants for 24 days. These colonies served as controls. During the 24-day period 170 plants were exposed to the virus-bearing colonies, and 176 plants to the virus-free colonies. The plants were kept under observation for a period of 2 months following the last exposure. Of the 170 plants on which virus-bearing colonies fed, 169 came down with stunt. One plant escaped infection.

TABLE 2
*Efficiency of *Baldulus maidis* as a Vector of Stunt*

Day of exposure	Transmission records																			
	5/31	6/1	6/2	6/3	6/4	6/5	6/6	6/7	6/8	6/9	6/10	6/11	6/12	6/13	6/14	6/15	6/16	6/17	6/18	6/19
Insect no. 1.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 2.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 3.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 4.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 5.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 6.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 7.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 8.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 9.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 10.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
" " 11.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
" " 12.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = plant diseased.
 - = plant healthy.
 * = plant dead.
 0 = insect dead.

All plants exposed to virus-free colonies remained healthy. The experiment showed that *B. maidis* was an efficient vector.

In another test of efficiency, 10 adult leafhoppers that had been hatched and reared on stunt corn plants were placed in separate lantern globe cages and transferred to healthy young corn plants daily for a period of 26 days. Two insects, hatched and reared on healthy corn plants, served as controls. The experiment was ended 62 days after the last plants were exposed. Results are shown in Table 2.

Each of the 10 leafhoppers from diseased corn transmitted stunt to each of the 150 plants on which they fed during the first 15 days of the test. On the 16th day insect no. 1 did not transmit. Five of the plants exposed to viruliferous insects during the last 11 days of the test died without showing

symptoms and before symptoms could be expected. The other 89 plants became diseased. Of the 240 plants that survived, 239, or about 99½ per cent, became diseased. The 48 plants exposed to control insects remained healthy. The experiment proved that *Baldulus maidis* was a highly efficient vector.

Stunt not Transmitted Mechanically

Many attempts were made to transmit stunt by the rubbing method using juice from diseased corn leaves. These invariably failed. One experiment will be described.

Twenty-two young corn plants of the sweet corn variety Golden Bantam, and 22 young corn plants of the field corn variety New Jersey hybrid no. 2, were separated into two groups. One plant of each variety was placed in the first group and 21 plants of each variety in the second group. The leaves of all plants were dusted lightly with carborundum. The leaves of the 2 plants in the first group were then rubbed with juice from a healthy corn plant by means of a cloth covered spatula. The leaves of the 42 plants in the second group were rubbed with juice from leaves of a stunt corn plant by means of a similar spatula. The plants were started in 4-inch pots and later transferred to 6-inch pots in which they grew vigorously. They were kept under observation for 3 months after inoculation. None developed symptoms of stunt. It was concluded that stunt virus probably can not be transmitted mechanically by means of juice from diseased leaves.

Stunt not Transmitted through Corn Seeds

Two lots of corn seeds were harvested from badly diseased plants of the variety New Jersey hybrid no. 2. From the first lot 35 plants and from the second lot 159 plants were grown to maturity. The first lot was kept under observation for a period of 102 days after planting and the second lot for a period of 97 days. The seeds were sown in flats. After the plants grew they were transferred, first to 4-inch pots and later to 6-inch pots. At no time did they show any symptoms of stunt. It was concluded that stunt rarely if ever is transmitted through corn seeds.

Stunt not Transmitted by Peregrinus maidis

Although it was evident that corn stunt was distinct from corn stripe, transmitted by *Peregrinus maidis* (Ashm.),¹³ it seemed desirable to determine whether or not the vector of stripe also might serve as a vector of stunt. *Peregrinus maidis* usually is not numerous on corn in New Jersey. However, it occurred in small numbers in corn fields in the vicinity of Princeton, New Jersey, during August of 1946. A few leafhoppers of this species were caught

and allowed to multiply on healthy corn in cages.* From time to time colonies were used in attempts to transmit stunt. Since they did not transmit the disease, only one experiment will be described.

Two hundred virus-free individuals of *Peregrinus maidis* were allowed to feed on stunt corn plants from April 14 to May 5. Then 2 colonies of adults, consisting of 47 and 50 individuals respectively, and 1 colony of nymphs of different ages consisting of 50 individuals, were taken from the stunt corn and transferred at daily intervals to healthy young corn plants for a period of 2 weeks. The healthy corn plants on which the colonies fed were kept under observation for 2 months following exposure. All plants remained healthy. It was concluded that *Peregrinus maidis* can not transmit the stunt disease.

Stunt not Transmitted by the Aster Leafhopper

The aster leafhopper *Macrosteles divinus* (Uhl.) occurs on corn over large areas. Hence an attempt was made to determine whether or not it could transmit stunt. About 350 aster leafhoppers, consisting of nymphs and adults, were placed in a large glass and screen cage containing stunt corn plants. After feeding on the plants for 2 weeks, 2 colonies consisting of 150 leafhoppers each were placed in lantern globe cages and transferred daily for a period of 2 weeks to healthy young corn plants growing in 4-inch pots. Most of the pots contained 3 plants, but a few contained 4. The exposed plants were kept under observation for 2 months following the last exposure. When they grew too large for 4-inch pots, they were transferred to 6-inch pots. At the end of the 2-month period it was found that 89 exposed plants were still living and that all were healthy. Since the experiment was carried out under conditions highly favorable for transmission of stunt by *Baldulus maidis*, it was concluded that *Macrosteles divinus* can not transmit stunt disease.

Susceptibility of Corn Varieties

New Jersey hybrid no. 4 was used in the first experiments with stunt. In later experiments New Jersey hybrid no. 2 and Golden Bantam were added. All were highly susceptible. Thirty-one other varieties were tested.† They were used in two experiments. In each experiment 2 pots of corn of each variety were exposed to viruliferous leafhoppers for 2 days. A third pot of corn of each variety was exposed to virus-free leafhoppers for the same period of time and served as a control. Usually each pot held 2 plants. After exposure all plants were freed of insects and were placed in a greenhouse. The plants were started in 4-inch pots but were soon transferred to 6-inch pots. In the latter

* The writer is indebted to Dr. Paul W. Oman of the United States Department of Agriculture, Washington, D. C., for identification of this leafhopper.

† The writer is indebted to Dr. Edwin J. Wellhausen of the Rockefeller Foundation, Mexico D. F., Mexico, for seed of these varieties.

they grew to maturity. Every plant, except one, that was exposed to viruliferous insects came down with stunt. All control plants remained healthy. The fact that one plant escaped infection was not believed to be significant. The experiment showed that all varieties tested were highly susceptible. Dr. Wellhausen has informed me that the 31 varieties represented only 5 or 6 different types of corn. A more extensive test might reveal resistant types.

Host Range of Stunt

It was expected that corn stunt would go to many different grasses since corn was so highly susceptible. However, of all the grasses tested only teosinte, a plant long recognized as a close relative of corn, took the disease. Grasses and other plants listed in Table 3 were exposed to very large colonies of viruliferous insects for the periods shown (Table 3). Four transmission experiments were made with teosinte. All plants exposed for 10 days, 4 of the 5 exposed for 7 days, 7 of the 10 exposed for 5 days, and 6 of the 12 exposed for 3 days, came down with stunt. The other plants remained healthy. The last experiment with teosinte will be described in detail.

Twelve healthy, young teosinte plants and 12 healthy, young corn plants of the variety New Jersey hybrid no. 2 were exposed from May 17 to May 20. Twelve other healthy, young teosinte plants and 12 other healthy, young corn plants were exposed to nonviruliferous leafhoppers for the same period and served as controls. The experiment was ended on July 8, 52 days after the plants were first exposed. Six of the teosinte plants, as reported above, and all of the corn plants on which viruliferous insects fed came down with stunt. The other teosinte plants and all control plants remained healthy. The experiment showed that teosinte was susceptible but less susceptible than corn. Virus-free leafhoppers allowed to feed on diseased teosinte plants for one week transmitted stunt to corn. The symptoms in teosinte were much like those observed in corn. Chlorotic spots first appeared on the bases of leaves. In leaves produced after those that showed the first symptoms, progressively larger areas were affected until finally entire leaves were chlorotic throughout. A section of a leaf from a diseased teosinte plant is shown beside a section of a comparable leaf from a healthy plant in Figure 5. The disease was in the striping stage in the section presented.

Several of the grasses listed in Table 3, *Coix Lacryma-Jobi*, *Euchlaena mexicana*, *Polyloca macrophylla*, *Tripsacum dactyloides* and *T. floridanum*, belong in the Maydeae. They are among the closest relatives of corn. Although, except in the case of teosinte (*Euchlaena mexicana*), only 3 plants of each of the species were exposed, it is believed that the tests were adequate for the plants were large and were stooling freely. Each of the *Tripsacum* plants presented at least 50 growing points to the vectors, the *Polyloca* plants about 15 growing points each, and the *Coix Lacryma-Jobi* plants about 6 growing points each.

TABLE 3
Plants Exposed to Viruliferous Leafhoppers

No. of plants	Kinds of plants	Period of exposure
	Monocotyledonous plants	
7	Sugar cane plants, variety unknown	10 days
2	" " " , variety CO-290*	7 "
4	" " " , variety CP-36-150*	7 "
4	Sudan grass plants	1 "
54	" " "	2 "
4	" " "	5 "
5	" " "	14 "
8	" " "	18 "
6	" " "	21 "
4	" " "	35 "
4	" " "	37 "
3	Sorghum plants	14 "
3	" "	18 "
30	Wheat plants	2 "
30	Barley plants	2 "
30	Oat plants	2 "
30	Rye plants	2 "
2	<i>Coix Lacryma-Jobi</i> plants	3 "
1	" " "	42 "
4	<i>Euchlaena mexicana</i> (teosinte) plants	10 "
5	" " "	7 "
10	" " " "	5 "
12	" " " "	3 "
2	<i>Tripsacum dactyloides</i> plants**	2 "
1	" " "	7 "
2	" <i>floridanum</i> plants**	2 "
1	" " "	7 "
2	<i>Polytoca macrophylla</i> plants**	2 "
1	" " "	7 "
	Dicotyledonous plants	
13	Carrot plants	10 days
10	" "	27 "
3	Sugar beet plants	10 "
4	" " "	33 "
9	Calendula plants	5 "
4	" "	31 "
3	Parsley plants	32 "

* The writer is indebted to Dr. *Sidney F. Sherwood* of the Division of Sugar Plant Investigations, Bureau of Plant Industry, U. S. Department of Agriculture, Beltsville, Md., for sugar cane cuttings of these varieties.

** The writer is indebted to Prof. *Paul C. Mangelsdorf* of Harvard University, Cambridge, Mass., for plants of these species.

The other grasses listed are not so closely related to corn as those in the May-deae. They were tested because of their economic importance.

The vector did not thrive on the cereal grasses, on sugar cane, or on sorghum. A considerable number of leafhoppers lived for about 3 weeks on each of these plants but the colonies dwindled rapidly. They deposited eggs in both sugar cane and sorghum. The eggs hatched, but the insects did not reach the adult

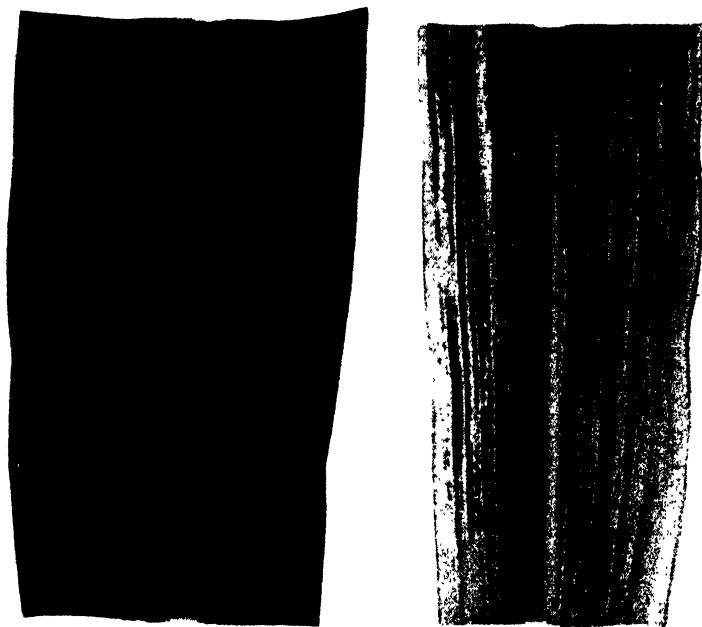


FIG. 5. Sections of leaves of teosinte. The section at the left is from a healthy plant; that at the right is from a stunt plant and shows a striping pattern. Photographed by J. A. Carlile.

stage on either. Sudan grass seemed to be the most favorable leafhopper host of all the grasses tested except corn and teosinte. Hence, a considerable effort was made to transmit stunt to Sudan grass. Since no symptoms were obtained it was thought that Sudan grass possibly might be a masked carrier. Virus-free leafhoppers were placed on 2 of the plants exposed for 21 days and on 2 of the plants exposed for 35 days. The leafhoppers were put on the plants about six months after they had been exposed. After staying on the Sudan grass plants for a week the insects were tested on corn. All corn plants remained healthy. It was concluded that Sudan grass was not a masked carrier. Similarly, virus-free leafhoppers were allowed to feed for a week on one of the CO-290 and one

of the CP-36-150 sugar cane plants that had been exposed for 7 days, about eight months after exposure. These insects did not transmit stunt to corn. It was concluded that the sugar cane plants also were not masked carriers. No attempt was made to obtain virus from plants of other species that failed to show symptoms.

None of the dicotyledonous plants listed in the table became diseased. They were used in transmission experiments because they proved to be fairly good host plants of the leafhopper. Adults were able to live for more than a month on either sugar beets or *Calendulas*. They deposited numerous eggs in the latter. However, the young were unable to live for more than a few days on

TABLE 4

Length of Incubation Periods of Stunt in Corn of the Variety New Jersey Hybrid Number 2

		Incubation periods																									
Days	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	
Experiment I	2			3	4	6	6	4	4	3	3		3	2	2	2	2	2	3	1		1	3	1	1		
Experiment II								1				1	1	1	1	1	1	1	1	1		1	2	2	4	2	
Days	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72						
Experiment I								1			1	1	1	1								Total 65 plants					
Experiment II	2	1	1	2	2	4	1	1			1	1			1	3		1	1		1	Total 44 plants					

Calendulas. Nymphs hatched on Calendulas and transferred to corn, lived and matured. Adults were able to live for two weeks on both carrot and parsley plants.

Incubation Period in Corn

Stunt virus was found to have a surprisingly long incubation period in corn. The period was so long, in fact, that plants used in early transmission experiments were discarded before they had had time to come down. Typical incubation periods are listed in Table 4. The table shows the lengths of the incubation periods in 109 corn plants used in two experiments in which all plants that were exposed became diseased. The first experiment was carried out in autumn when light was good and greenhouse temperatures moderately high. The second experiment was carried out in winter, when light was poor and temperatures in the greenhouse in which plants were held varied from 70° to 75° F. Plants in both experiments were exposed to colonies of about 50 viruliferous leafhoppers.

The incubation periods were considerably shorter under autumn than under

winter conditions. However, there was great variability in the lengths of the periods in different plants during both seasons. The shortest incubation period shown was 26 days for each of 2 plants in experiment I; the longest was 72 days for a plant in experiment II. Less than one-third of the plants came down within 35 days after exposure and less than one-half within 42 days. No corn plant used in any experiment carried out during the past two years has shown an incubation period shorter than 26 days.

Distance between Area Inoculated and Area Showing First Symptoms

When corn stunt was found to have a long incubation period in the corn plant, an experiment was made to determine in what leaf above that which was inoculated the first symptoms of disease might be expected to appear. In this experiment about 2 inches of the tip of a single leaf, usually the third or fourth leaf of a young plant of N. J. hybrid no. 2, was inoculated by exposing it to 30 virus-carrying leafhoppers for one day. The exposures were made during the month of May when conditions for transmission were very favorable. It was not until the 46th day after the last exposure that all of the plants showed symptoms.

In 7 of the plants chlorosis first appeared on the fifth leaf, in 12 on the sixth leaf, in 9 on the seventh leaf, and in 2 on the eighth leaf above that which was inoculated. There was very little chlorosis, usually only a few spots or short stripes, on one or both sides of the base of the first leaf to show symptoms. Conspicuous striping and general chlorosis appeared in leaves produced further up the stalk. Even when the disease appeared in the fifth leaf above that inoculated there was a long distance between the area on which the vector fed and the area that showed the first symptoms. The distance between these areas probably was correlated with the length of the incubation periods in the plants.

*Incubation Period in *Baldulus maidis**

A number of experiments were carried out which proved that stunt virus required a long incubation period in the leafhopper before it could be transmitted to healthy corn plants. Twenty-four virus-free cultures, varying in size from one to 50 insects, were allowed to feed on stunt corn plants for varying periods of time, and were then transferred successively at daily intervals to healthy corn plants for periods of 23 or more days. Exposed plants were held under observation for 60 days or longer. The tests were made in 9 different experiments, distributed through the four seasons of the year. Nine virus-free control cultures, consisting of colonies of the same sizes as those used in the tests, were confined on healthy corn plants, while the 24 cultures mentioned above were kept on diseased plants. They were then transferred successively at daily intervals to healthy corn plants. The plants exposed to control insect cultures were held for the same periods of time and under greenhouse conditions similar to those

under which the test plants were kept. All plants on which control cultures fed remained healthy. Details of results obtained with cultures that had fed on diseased corn are shown in Table 5.

All cultures except 1, 10, 12, 16, 17, 18, 19, 20, 21, and 22 were allowed to feed on stunt plants for one day only. Culture 1 fed on diseased plants for 10 days, culture 10 for 7 days, culture 16 for 2 days, cultures 12 and 17 for 3 days, cultures 18, 19, and 20 for 4, 5, and 6 days, respectively, and cultures 21 and 22 for 40 days. Since cultures of 10 or more insects always picked up virus during one day of feeding, it was assumed that virus was picked up on the first day when the period of feeding exceeded one day. Calculation of incubation periods listed for the 10 cultures mentioned above was based on that assumption. No assumption was involved in calculating the incubation periods for the other 14 cultures. Cultures 10 and 15 to 22, inclusive, consisted of one-day old nymphs when placed on stunt plants. Cultures 12, 23, and 24 were of nymphs in the second and third instar stages. All other cultures consisted of adults when they were placed on diseased plants. Results shown in the table proved that both nymphs and adults picked up virus and that adults transmitted it. Results not shown in the table proved that nymphs also could transmit the virus.

Cultures 1 to 24 show incubation periods varying from 14 to 32 days. It will be noted that tests were carried out during periods beginning February 5, April 27, June 13, June 18, July 3, July 12, October 15, November 2, and December 13. As the season advanced the incubation period in the insect decreased from 32 days for the test begun February 5 to 14 days for two of the tests begun on June 18 and the test begun on July 3. This decrease in the length of the incubation period probably was correlated with increase of temperature in the greenhouses in which cultures were held. The houses were kept at 70° to 75° F. during February, March, and part of April. As the season progressed, outside temperatures rose above 75° F. and the temperatures in the greenhouses likewise rose. The lengths of incubation periods in cultures 11 and 12, used in tests begun July 12, were longer than those shown by the tests begun April 27, June 13, June 18, and July 3, although the temperatures prevailing during the time cultures 11 and 12 were being tested undoubtedly were higher than those prevailing during the other four periods. It is possible that temperatures reached during the period beginning July 12 were so high that they retarded the processes involved in the incubation of virus in the insect. Individual insect cultures 13 and 14 were held at a constant temperature of 86° F. during the tests. These cultures gave incubation periods of 18 and 20 days, respectively. The results suggest that 86° F. was somewhat too high to give the minimum incubation period. Cultures 15 to 20, used in tests begun November 2, show incubation periods of 21, 15, 16, 20, 15, and 15 days, respectively. Al-

though there was considerable variability in the lengths of these periods, four of the six were rather short. This suggests that greenhouse temperatures in November were more favorable for incubation in the insect than were midsummer temperatures. As was to be expected, cultures 21 and 22 transmitted during the first day they were taken from diseased plants and on each day of the test thereafter. The virus, no doubt, had completed its incubation in these insects long before the insects were transferred to healthy plants. The fact that cultures 23 and 24, used in tests begun December 13, gave incubation periods of 21 days each, suggests that greenhouse conditions during the last half of December and early in January were somewhat unfavorable for incubation in the insect.

The minimum incubation periods of 14 days shown in 3 of the tests (cultures 7, 8, and 10), are the longest minimum incubation periods that have been reported for any plant virus in an insect vector that has been adequately tested. It is possible that in further studies temperatures and other conditions more favorable than those that prevailed while cultures 7, 8, and 10 were being tested will be discovered and that incubation periods somewhat shorter than 14 days will be reported. However, it is not believed that periods substantially shorter than 14 days will be found.

The table shows that shortly after completion of the incubation period several cultures (4, 5, 7, 8, 11, 15, 16, 17, and 19) transmitted to fewer plants than they did somewhat later. It was observed that the plants which came down following exposure to insects in which virus had recently completed its incubation were less severely affected during onset stages than those exposed to the same insects later. This increasing certainty of infection and degree of severity in the disease produced by insects in which ability to transmit was recently acquired, suggest that the dose of virus transmitted by the colonies increased with time. This increase probably resulted from completion of incubation periods in individual insects.

*Retention of Stunt Virus by *Baldulus maidis**

No experiments were made for the express purpose of determining how long stunt virus might be retained by *Baldulus maidis*. However, information on this subject was furnished by experiments carried out for other purposes. Transmission records of cultures 6, 15, 23, and 24, mentioned in Table 5, show that each culture transmitted virus on the 46th day after it had fed on a diseased plant for one day. In two transmission experiments not reported here virus was retained for 76 and 88 days, respectively, by insect cultures that had been exposed to diseased plants for one day and transferred to healthy young plants at daily intervals thereafter. The data show that the insect retained stunt virus for long periods of time.

Correlation between Lengths of Incubation Periods in Insects and in Plants

The lengths of the incubation periods of stunt virus in *Baldulus maidis* and in corn plants show a striking contrast when compared with the incubation periods of streak and stripe viruses in their insect vectors and in corn. Streak virus has a very short incubation period in *Cicadulina mbila* Naude under moderate temperature conditions. Its minimum length, $\frac{1}{4}$ to $\frac{1}{2}$ day, is the shortest that has been reported for any virus in an insect vector. It is interesting that this virus also has an exceedingly short incubation period in the corn plant. Under favorable conditions its length is from 2 to $3\frac{1}{2}$ days, according to Storey.²² Stripe virus, on the other hand, has an incubation period of intermediate length in its vector, *Peregrinus maidis*. It also has an incubation period of intermediate length in the corn plant. The minimum lengths of the incubation periods in insect and in plant are about 4 days, according to Carter.⁷ It is obvious that there is a good correlation between the lengths of the incubation periods of these three viruses in their insect vectors and in corn.

After discussing evidence in support of and against the theory that certain plant viruses multiply in their insect vectors, Bowden² states: "The reason for the incubation period in vectors of this type of virus is still not known. The most probable explanation appears to be that it is the time necessary for the virus to penetrate the gut wall, pass through the blood and enter the saliva so that it is in a position to be injected into plants." If this were the true explanation, why should there be a correlation between the lengths of the incubation periods of these three corn viruses in their insect vectors and in corn? The three vectors, *Cicadulina mbila*, *Peregrinus maidis*, and *Baldulus maidis* are of the same order of size. There is no obvious reason why one of the viruses, the virus of corn streak, should require only one-half day to pass from gut to saliva in one leafhopper while another virus, the virus of corn stunt, should require 14 days, or 28 times as long, to make a similar trip in another leafhopper. The transfer from gut to salivary gland must, of course, be made and undoubtedly some part of the incubation period in each case must be allotted to this trip, but if only these transfers were involved it is difficult to believe that such different lengths of time would be required, or that the time required for the passages would be related in any way to lengths of incubation periods in corn plants. If, on the other hand, the incubation periods of viruses in insect vectors of this type are periods in which the viruses not only move in the insects, but multiply and are liberated from the cells in which they multiply in sufficient concentration for transmission to plants, it is easy to see why there should be a correlation between lengths of incubation periods in insect vectors and in plants. A virus that multiplies rapidly should have a short incubation period in both insect and plant. A virus that multiplies slowly should have a long incubation period in both insect and plant.

After noting the correlation between the lengths of incubation periods of the three corn viruses in their vectors and in corn, an attempt was made to determine whether or not this correlation extended to other viruses transmitted by specific leafhopper vectors. Determination of lengths of incubation periods both in vectors and in plants have been made under widely different conditions. In some instances the conditions have not been described. Hence, from the literature on plant viruses, it is not possible to obtain lengths of incubation periods that can be considered highly accurate either in insects or in plants.

TABLE 6
Incubation Periods of Viruses in Leafhopper Vectors and Plant Hosts

Virus	Plant	Vector	Incubation period in insect vector	Incubation period in plant host
Streak	Corn	<i>Cicadulina mbila</i> Naude	$\frac{1}{4}$ to $\frac{1}{2}$ day	3 days
Curly top	<i>Stellaria</i> <i>media</i>	<i>Eutettix tenellus</i> Baker	1 day	2 "
Stripe	Corn	<i>Peregrinus maidis</i> Ashm.	4 days	4 "
Mosaic	Winter wheat	<i>Deltocephalus</i> <i>striatus</i> (L.)*	5 "	9 "
Pseudo- rosette	Oat	<i>Delphax striatella</i> Fall.	6 "	8 to 10 days
Yellow dwarf	Crimson clover	<i>Aceratagallia san-</i> <i>guinolenta</i> Prov.	6 "	about 10 days
Aster yellows	Aster	<i>Macrostelus divisus</i> (Uhl.)	9 "	9 days
Rice stunt	Rice	<i>Nephotettix apicalis</i> Uhler	10 "	5 "
Corn stunt	Corn	<i>Baldulus maidis</i> (De L. and W.)	14 "	26 "

* Only 1st, 2nd, and 3rd instars can pick up winter wheat mosaic virus.

Nevertheless, the lengths of incubation periods reported in a number of instances are sufficiently accurate to show that the correlation noted above holds for several other plant viruses. The lengths of incubation periods listed in table 6 are the minima that have been reported for tests made under ordinary greenhouse and laboratory conditions. Incubation periods of six different viruses, in addition to the three affecting corn, are given in the table.

The virus of curly top of sugar beets^{6,20} is like that of corn streak²² in having a very short incubation period in both vector and plant. At temperatures of about 100° F. it has an incubation period of only four hours in the leafhopper, but at ordinary laboratory temperatures the period is approximately one day. The viruses of corn stripe,⁷ winter wheat mosaic,²⁹ pseudo-rosette of oats,²⁸ and

potato yellow dwarf,⁸ have incubation periods of 4, 5, 6, and 6 days, respectively, in their vectors, and 4, 9, 8-10²⁶ and about 10 days, respectively, in their plant hosts. These incubation periods are of intermediate lengths both in the insects and in the plants. The viruses of aster yellows, rice stunt,¹⁰ and corn stunt are shown in the table to have incubation periods of 9, 10, and 14 days, respectively, in their vectors, and 9, 5, and 26 days, respectively, in their plant hosts. In 1926 aster yellows virus was reported to require minimum incubation periods of 10 and 12 days,¹⁴ respectively, in the aster leafhopper and the aster plant. More recent experiments, not previously reported, have given minimum incubation periods of 9 days in the insect and 9 days in the plant. These shorter periods were obtained under highly favorable temperature conditions. The only virus among those listed in the table that is reported to have a longer incubation period in its vector than in its plant host is that of rice stunt.¹¹ It should be pointed out that the incubation period of this virus in its vector has not been accurately determined, due in part no doubt to the fact that a minimum period of 3 days was required for the vector to pick up this virus. Rice stunt probably belongs in the group of viruses having incubation periods of intermediate lengths. The viruses of aster yellows and corn stunt have long incubation periods in both vectors and plant hosts. In general, the table shows that there is a good correlation between the lengths of incubation periods in leafhopper vectors and in plants. The correlation is believed to support the theory that plant viruses multiply in leafhoppers.

DISCUSSION

When culture of corn spread from America to other parts of the world the corn plant became subject to several different virus diseases. In Africa it was attacked by streak²³ and a mild mottling disease.²⁴ In Hawaii,^{13,15} Guam,²⁸ Cuba,¹⁶ Puerto Rico,⁸ Trinidad,⁵ and Africa²⁸ it contracted a disease known as stripe. In Australia¹⁹ it was affected by Wallaby Ear disease. As sugar cane mosaic spread throughout the cane-growing world, it went to a limited extent from cane to corn in many places.⁴ Celery mosaic, caused by a strain of cucumber mosaic virus, occurs naturally on corn in Florida.²⁷ However, in the United States corn has remained generally free from virus diseases. Corn stunt, which has recently appeared in California and Texas, may or may not spread to other parts of the country. Doubtless much will depend on the ability of the vector to migrate northward.

Little is known regarding the present distribution of corn stunt. Information received by letter from Dr. *I. E. Melhus* indicated that it is prevalent in Guatemala and other Central American countries. It probably occurs in Mexico and there is a possibility that it may occur in Cuba and Puerto Rico. *Stahl*²¹ reported that corn near the place where some of his experiments were

carried out was "heavily infested with the corn leafhopper (*Peregrinus maidis* Ashm.) as well as a small yellow leafhopper (*Cicadula maidis* De Long)". The small yellow leafhopper referred to was undoubtedly *Baldulus maidis*, the vector of stunt. Stahl stated that the disease with which he worked was transmitted by *Peregrinus maidis* but does not describe his experiments in detail. It seems likely that both stripe and stunt may occur in Cuba. Cook,⁸ working in Puerto Rico, made a cytological study of tissues affected by a corn virus disease which he presumed to be stripe. At the end of the paper he states "Intracellular bodies were not seen". Since intracellular bodies are associated with stripe but not with stunt, Cook's statement suggests that he may have been working with the latter disease, and hence, that stunt may occur in Puerto Rico. There apparently is no evidence that it occurs in other parts of the world.

The corn stunt virus seems to be highly specific for the leafhopper *Baldulus maidis* and for corn and its close relative teosinte. The vector also seems highly specific for corn and teosinte. However, only a relatively small number of plants and insects have been tested as hosts of the virus, and only a small number of plants as hosts of *Baldulus maidis*.

Stunt is severe on both corn and teosinte. However, because of its long incubation period it seldom appears in plants that are less than 30 to 50 days old. It typically is a disease of plants that are approaching maturity. The relation of the virus to its vector is characteristic for yellow type diseases.

SUMMARY

1. Symptoms of corn stunt disease as they occur in greenhouse cultures are described.

2. Time required for development of *Baldulus maidis* from egg to adult is recorded. Efficiency of this vector in transmission of stunt was found to be very high.

3. Stunt was not transmitted mechanically by means of juice from diseased plants. It was not transmitted by *Peregrinus maidis*, the vector of corn stripe, or *Macrosteles divinus*, the vector of aster yellows. It was not transmitted through seeds of diseased plants.

4. Some 34 varieties of corn that were tested proved highly susceptible to stunt. Teosinte also proved susceptible. Four other grasses related to corn, *Coix Lacryma-Jobi*, *Polytoca macrophylla*, *Tripsacum dactyloides*, and *T. floridanum*, were found to be immune. Sugar cane, Sudan grass, sorghum, wheat, barley, oats, and rye also were immune. Carrot, sugar beet, Calendula, and parsley plants, although acceptable to the vector as food plants, were immune.

5. Stunt virus was found to have a long incubation period in corn. The minimum found was 26 days. The first symptoms of stunt in young corn plants developed in the fifth to the eighth leaf above that inoculated. The long distance between the point of inoculation and the area first affected by chlorosis is believed to be related to the long incubation period in the plant.

6. The minimum incubation period of corn stunt in the insect vector was 14 days. This is the longest minimum incubation period reported for any plant virus in an insect vector where a careful study of length of incubation period has been made.

7. It was pointed out that there is a correlation between the lengths of the incubation periods of the three viruses causing streak, stripe, and stunt of corn in their specific insect vectors, and the lengths of the incubation periods of these viruses in corn.

8. A similar correlation was shown to apply to several other plant viruses that are transmitted by leafhoppers. It was suggested that the correlation may depend on the rates at which the different viruses multiply and, hence, supports the theory that plant viruses multiply in leafhopper vectors.

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ON THE NATURE OF THE INTERACTION BETWEEN ACTOMYOSIN AND ATP

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There is at present considerable interest in the nature of the interaction between adenosine triphosphate (ATP) and actomyosin (Weber's myosin). This interest originates in experiments which Engelhardt, *et al* (5) performed on actomyosin threads manufactured by squirting a thin stream of concentrated protein solution into water. These investigators found that ATP induces an increase in tensile strength of actomyosin threads made in this manner. Subsequently, Szent-Györgyi and his collaborators (8) have reported that threads of actomyosin which are floating freely in water "contract" upon treatment with ATP. Furthermore, Buchthal (2) has emphasized the possible importance of the ATP-actomyosin interaction in the living muscle cell. He maintains that ATP can produce a contraction in a single muscle fiber. This contraction is associated with an action potential and is accompanied by a reversible decrease in birefringence of the fiber. Indeed, Buchthal (1) has stated that the "breakdown of ATP is the reaction nearest in time to the physical process of contraction."

Unfortunately, agreement is not general on the nature of the effect of ATP on the actomyosin molecule. Thus, in a study of actomyosin dispersed in KCl solution, rather than in the form of threads, Needham and co-workers (3) observed that both ATP and high concentrations of KCl bring about a drop in viscosity and flow birefringence of the protein solution. They concluded that these changes are the result of a coiling up of the actomyosin molecules. Contrariwise, Szent-Györgyi considered quite similar data to indicate that both ATP and high KCl concentrations effect a dissociation of the dispersed actomyosin molecules into actin and myosin.

Induction of coiling in actomyosin by ATP is consistent with the notion that the ATP-actomyosin interplay is of fundamental importance in muscular contraction. However, if ATP produces dissociation in actomyosin, it is not easy to understand the rôle that this interaction might play in contraction of the muscle cell.

To obtain information of possible value for a clearer understanding of the ATP and KCl effect, we have undertaken the light-scattering and electron microscope studies described here in a preliminary report.

The actomyosin used in our experiments has been prepared by a method slightly modified from that of Greenstein and Edsall (6). The preparation was purified by ultracentrifugation at 28,000 rpm.

The light-scattering measurements were made with a simple apparatus which employs the 546-m μ line of a mercury vapor lamp as light source and a photomultiplier tube for registering intensities of scattered light. We have determined the intensities of light scattered in the horizontal plane at angles of 45° and 135° with a beam of light transmitted through a 1% solution of actomyosin in 0.5 molar KCl and 0.09 molar KH₂PO₄-K₂HPO₄ buffer at pH 6.8.

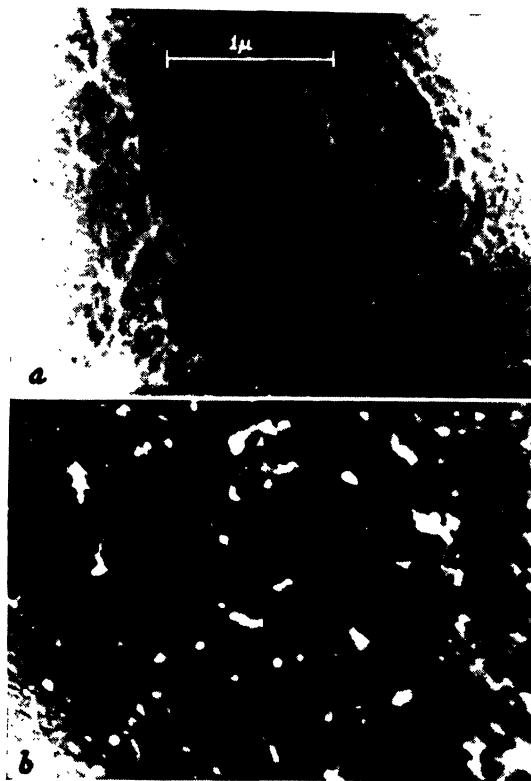


FIG. 1

In 10 observations on three preparations of actomyosin, an average value of 3.4 was obtained for the ratio of the forward to backward scattering at the angles mentioned.

Debye (4) has pointed out that measurements of this ratio yield information concerning the size, shape, and behavior of macromolecules comparable in size with the wave length of light. Furthermore, Oster (7) has determined the limiting values of the ratio to be expected for macromolecules which are in the form of spheres, random coils, or stiff rods. In the light of Oster's

calculations for the angles used here, the value of 3.4 which we have found for actomyosin indicates that this protein has a configuration intermediate to that of a stiff rod or random coil; that is, the actomyosin particles in 0.5 molar KCl at pH 6.8 are slightly coiled.

After addition of ATP in quantity sufficient to make its final concentration 1% in a solution of actomyosin in 0.5 molar KCl and buffer at pH 6.8, it was found that the ratio had increased to 4.7. This was observed in 5 experiments on two preparations of actomyosin. On the basis of the theory of light scattering (see 7) one should expect the ratio to decrease if the actomyosin were dissociated by ATP. However, the increase in ratio we have observed is entirely consistent with an increase in coiling of the actomyosin.

No change in pH of the actomyosin solution after addition of ATP was detectable as measured by glass electrode. Tenfold dilution of the ATP-actomyosin solution with 0.5 molar KCl did not alter the ratio of 4.7, nor was there a change in light transmission of the actomyosin solution after addition of ATP. The transmission was measured in a Klett-Summerson colorimeter using a blue filter. The latter finding may be interpreted as indicating that the change in ratio is not the result of aggregation or coagulation of the actomyosin.

Addition of KCl to actomyosin solution over a concentration range of 0.5 to 2 molar KCl failed to alter the ratio at the previously stated angles. This result, obtained in 5 experiments done on two preparations, constitutes good evidence that the actomyosin does not dissociate at higher concentrations of KCl. It is, rather, an indication that there is no change in the size or shape of the protein as a result of increasing the concentration of KCl. Thus, the drop in viscosity of actomyosin solution observed at high KCl concentrations demands an explanation different from those previously offered. It is conceivable that the decrease in viscosity is related to an electroviscous effect and is associated with the adsorption of potassium by actomyosin.

Electron micrographs demonstrate an effect of ATP on actomyosin which is consistent with the preceding interpretation based on light-scattering data.

Samples of untreated actomyosin were prepared by placing a drop of 0.01% actomyosin solution in 0.5 molar KCl on a collodion film supported by a copper screen. The sample was allowed to dry and was washed with distilled water to remove readily soluble salts. The specimen was then gold shadow-cast by the method of Williams and Wyckoff (9) to enhance photographic contrast. Fig. 1a is a representative picture of such a sample taken in an RCA console model electron microscope. The typical entangled, fibrillar structure of actomyosin is illustrated, along with the characteristically wide distribution of particle lengths.

Similar samples were then prepared from solutions of 0.01% actomyosin in 0.5 molar KCl to which ATP had been added in sufficient quantity to make its

final concentration 0.01%. Fig. 1b is a picture of such an ATP-treated specimen. As can be seen in the figure, the bulk of the elongated fibrils of actomyosin have been changed into shortened, thickened irregularly shaped forms. They are of different sizes, as is to be expected from the wide distribution of particle size in the untreated specimen.

It was impossible to study the effect of varying KCl concentrations on actomyosin in the electron microscope. The changes in salt concentration which occur in the preparation of the sample make it impossible to know with any accuracy the effective KCl concentration.

Thus, we have obtained evidence from light-scattering observations which indicates that actomyosin dispersed in KCl solution has the configuration of a slightly coiled particle. Increasing the concentration of KCl does not affect this configuration. On the other hand, as shown by light-scattering data reinforced by electron micrographs, ATP increases the degree of coiling of actomyosin, an effect which is compatible with a fundamental rôle in muscular contraction for this nucleotide-protein interaction. Further studies on both the ATP and KCl effects are in progress.

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IMMUNOCHEMICAL STUDIES ON TOBACCO MOSAIC VIRUS

V. THE SEROLOGICAL RELATIONSHIP TO THE AUCUBA AND J14D1 STRAINS

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The ability of tobacco mosaic virus to mutate and to form virus strains which are distinct biologically has been noted by many observers (1-3). The relative ease with which preparations of these strains can be obtained in purified form and their remarkable constancy of properties has allowed rather thorough and extensive amino acid determinations on the protein moieties of these strains (4-7). Rather marked differences have been noted among some strains which, from a biological consideration, appeared to be most distantly related to the type strain and relatively little chemical difference among those strains which more closely resembled the parent in biological activity (7).

The serological relationship of the type strain, tobacco mosaic virus, to the Holmes' rib-grass, has been studied by means of the quantitative precipitin reaction. This strain differs rather markedly, biologically, from tobacco mosaic virus (8) and has been found to have certain marked chemical differences (5-7, 9), especially with reference to the serologically important amino acids, histidine and tyrosine (10). The existence of rather well defined and independent immunological determinants, as well as common antigenic determinants, has been established (11). Serological studies on many of the other strains of tobacco mosaic virus have been carried out only by semi-quantitative procedures.

The present communication deals with two mutants of tobacco mosaic virus which, from a consideration of their chemical composition (7), are more closely related to the type strain, namely, the green aucuba strain which was obtained by growing the yellow aucuba strain in a species of tobacco at an elevated temperature (12) and the J14D1 strain which arose by the spontaneous mutation of the J14 strain (13). With respect to the biological activity of these respective strains, it is noteworthy that the former differs but little from tobacco mosaic virus but the latter produces symptoms which are distinctive and markedly more severe (3). Although in early work Beale (14) and Chester (15, 16) found no serological difference between the aucuba strain and tobacco mosaic virus, it was established subsequently by Chester (17) and by Bawden and Pirie (18) that a qualitative serological difference does exist between these strains. More recently, Knight (19), by semiquantitative technics, determined the presence of antigenic differences between both the aucuba and the J14D1 strains and tobacco mosaic virus. In order to establish more definitely and completely the sero-

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logical relationship between these two strains and tobacco mosaic virus, analyses were carried out by following the quantitative precipitin reaction, a technic which has been utilized for many types of antigens (10, 20).

EXPERIMENTAL

Antigens: Tobacco mosaic virus (TMV) and green aucuba virus (GA) were used for both the immunization of rabbits and as test antigens. In addition, J14D1 was used also as a test antigen. These antigens were all prepared by differential centrifugation according to the methods used in this laboratory (21). The virus solutions were made up in physiological saline solution with no special precautions for maintaining sterility.

Antisera: Rabbit antisera were prepared against both TMV and GA by inoculating several groups of animals according to the schedule previously described (22). The sera of the individual rabbits of each group were pooled and sterility was maintained by passage through Berkefeld candles and storing at 6 C.

Precipitates and analyses: Precipitates for analyses, in duplicate, at arbitrarily selected points were obtained by allowing each of the test antigens to react with diluted anti-TMV serum in conical graduated centrifuge tubes. The reaction mixtures were made up so that the total amount of nitrogen carried down in the precipitates ranged from 0.02 to 0.08 mg. The procedures involved in the determination were essentially as previously described (22).

Absorptions: Two methods were carried out. One, listed as procedure A, consisted in adding an amount of antigen roughly calculated to precipitate all the antibody capable of combining, and the other, listed as procedure B, entailed an exhaustive absorption by the addition of small amounts of antigen to the anti-serum, with subsequent removal of each precipitate, until the further addition of antigen failed to cause any further precipitation. The procedures for typical experiments are described below.

(A) To 2 ml of anti-TMV serum was added 19.3 mg GA nitrogen contained in 13 ml of solution making a final dilution of serum of 1:7.5. The mixture was well stirred and allowed to incubate at 37 C for about 2 hours. After about 48 hours in the cold room at 6 C the precipitate which had formed was removed by centrifugation in the cold in a Swedish angle centrifuge. Arbitrary amounts of TMV, GA and J14D1 were added to separate portions of the supernate for the preparation of precipitates, and the mixtures were incubated and treated for analysis as described above.

The antibody from 2 ml of anti-GA serum was absorbed out by an identical procedure using 16.1 mg TMV nitrogen in 13 ml of solution. Portions of the supernate which was removed were also allowed to react with arbitrary amounts of TMV, GA and J14D1.

(B) One milliliter of GA solution containing 1.4 mg nitrogen was added to 2 ml of anti-TMV serum. The mixture was treated essentially the same as in procedure A. To the supernate was added an additional volume of GA solution containing 1.7 mg nitrogen and the mixture treated as before. To subsequent supernates were added additional quantities of GA antigen in amounts of 1.5 mg,

0.45 mg and 0.15 mg nitrogen. The last quantity of antigen failed to cause any demonstrable precipitate and brought the serum dilution to 1:6. For precipitate analyses there were added to the final supernate arbitrary amounts of TMV, GA, and J14D1 and the mixture treated as already described.

Likewise, to 2 ml anti-GA serum was added TMV in 1.1 mg, 1.1 mg, 1 mg, 0.69 mg and 0.15 mg nitrogen quantities, or until there no longer appeared any precipitate. To portions of the final supernate, which consisted of the original

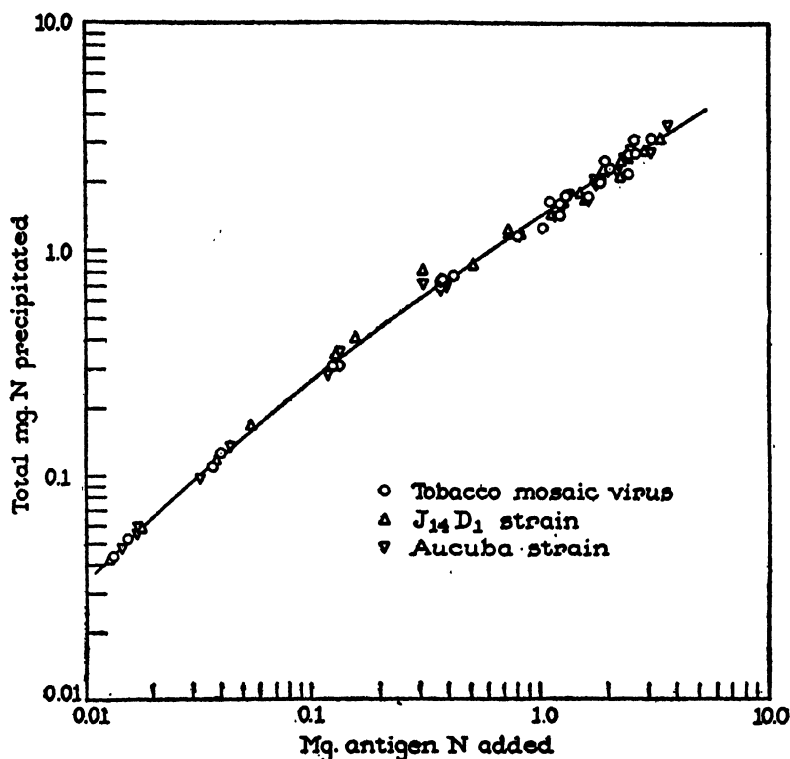


FIG. 1. Quantitative precipitin reaction between Tobacco Mosaic Virus and certain of its strains with anti-TMV Serum.

serum at a dilution of 1:6, were added TMV, GA and J14D1 for precipitation and the analyses carried out as above.

RESULTS

The data obtained from the quantitative precipitin reaction of TMV, GA and J14D1 with anti-TMV serum are shown in figure 1. The graph shows the amount of total nitrogen precipitated as the ordinate with the mg of added nitrogen as the abscissa, the values having been recalculated on the basis of 1 ml antiserum. With increasing amounts of antigen, the total nitrogen precipitated increases towards a maximum. No apparent difference exists between TMV

and the strains, GA and J14D1, with respect to the amount of total nitrogen precipitated. There is apparently but little difference in the antibody combining capacity of the heterologous antigen as compared to the homologous antigen. This method fails to indicate serologically independent specific determinants if present.

Tables I and II contain the absorption data. The quantitative analysis of the precipitates was not possible because of the small quantity resulting and the limitations of the method used. However, it is possible to draw a roughly quantitative relationship for antibody specific to both TMV and J14D1 remaining following absorption of an anti-TMV serum by GA. From both procedures it is possible to arrive at comparable conclusions. The antibody remaining showed a

TABLE I
Serum absorbed by a single dose of antigen

1 ML ABSORBED SERUM DILUTED	MG N ADDED PER ML UNDEL. ANTISERUM (CALC.)	TMV ABSORBED ANTI-GA (PPT.)	GA ABSORBED ANTI-TMV (PPT.)
TMV added to absorbed antiserum			
1:7.5	0.335	0	+
1:15	1.004	0	±
1:30	3.011	0	0
1:45	4.516	0	0
J14D1 added to absorbed antiserum			
1:7.5	0.315	±	±
1:15	0.945	0	0
1:30	2.835	0	0
1:45	4.442	0	0
GA added to absorbed antiserum			
1:7.5	0.345	+	0
1:15	1.035	0	0
1:30	3.105	0	0
1:45	4.554	0	0

greater combining capacity for the homologous antigen than for the heterologous antigen, as could have been predicted. Similar results and conclusions were obtained from TMV absorbed anti-GA serum.

This rather weak serological independence between TMV and its strains was again demonstrated in the experiment detailed in table III. By using a concentrated antigen, the final dilution of the anti-GA serum absorbed by TMV was 1:1.5. This enabled estimable quantities of precipitate to be formed in the precipitin procedure. Again the homologous antigen carried down appreciable quantities of antibody.

Interesting results were obtained with antisera which were not completely absorbed by the heterologous antigen. The data for a typical experiment is given

TABLE II
Serum exhaustively absorbed by antigen

1 ML ABSORBED ANTI-SERUM DILUTED	MG N ADDED PER ML UNDIL. ANTISERUM (CALC.)	TMV ABSORBED ANTI-GA (FFT.)	GA ABSORBED ANTI-TMV (FFT.)
TMV added to absorbed antiserum			
1:6	0.348	0	±
1:12	0.937	0	0
1:20	2.899	0	0
1:40	4.460	0	0
J14D1 added to absorbed antiserum			
1:6	0.328	±	?
1:12	0.882	0	0
1:20	2.730	0	0
1:40	4.620	0	0
GA added to absorbed antiserum			
1:6	0.358	+	0
1:12	0.828	0	0
1:20	2.760	0	0
1:40	4.600	0	0

TABLE III
Absorption of anti-GA serum by a single dose of concentrated TMV

1 ML ABSORBED SERUM DILUTED	MG N ADDED PER ML UNDILUTED ANTISERUM (CALC.)	MG TOTAL N IN FFT./ML UNDIL. SERUM
TMV added to absorbed antiserum		
1:1.5	0.017	0
1:3	0.033	0
1:6	0.121	0
1:10	0.357	0
J14D1 added to absorbed antiserum		
1:1.5	0.016	±
1:3	0.038	?
1:6	0.126	0
1:10	0.357	0
GA added to absorbed antiserum		
1:1.5	0.017	0.027
1:3	0.035	0.045
1:6	0.124	0.069
1:10	0.368	0.027

in table IV. Each of the three test antigens gave a measurable amount of precipitate when added to anti-TMV serum from which there had not been complete absorption of antibody specific to the absorbing antigen, GA. The greater

TABLE IV
Precipitates from anti-TMV serum partially absorbed by GA

2 ML ABSORBED SERUM DILUTED	MG N ADDED PER ML UNDILUTED ANTISERUM (CALC.)	MG TOTAL N IN PPT./ML UNDIL. ANTISERUM
TMV added to partially absorbed antiserum		
1:7.5	0.056	0.084
1:10.2	0.166	0.176
1:17.5	0.497	0.464
1:23.2	1.256	0.426
1:46.5	2.640	0.116
J14D1 added to partially absorbed antiserum		
1:7.5	0.056	0.075
1:10.2	0.167	0.176
1:17.5	0.501	0.459
1:23.2	1.265	0.463
1:46.5	2.656	0.126
GA added to partially absorbed antiserum		
1:7.5	0.062	0.017
1:10.2	0.202	0.022
1:17.5	0.605	0.394
1:23.2	1.530	1.412
1:46.5	3.213	2.339

TABLE V
Effect of antigen on antibody—antigen complex

GA		TMV	
mg TMV N added	Σ mg N ppt.	mg TMV N added	Σ mg N ppt.
Anti-TMV Serum Precipitate with			
Control	0.050	Control	0.041
0.022	0.059	0.024	0.034
0.045	0.066	0.20	0.043
0.089	0.092	0.64	0.024
0.20	0.130	0.96	0.014
0.50	0.128	1.52	0.011
3.0	0.125	2.0	0.009

amount of total nitrogen was precipitated by the addition of a further quantity of the heterologous, absorbing antigen, and not by the homologous antigen, TMV. Several experiments corroborated these findings.

Precipitates of GA with its specific antibody were allowed to react with increasing amounts of TMV by incubating the well suspended precipitate with the heterologous virus for about 2 hours followed by storing at 6 C for about 48 hours. Table V indicates that bound GA-specific antibody could combine with additional serologically related antigen. The amount of total nitrogen precipitated passed through a maximum and then diminished. This is in agreement with the concept that the antigen-antibody complex becomes increasingly soluble in the region of antigen excess (10). From the same table it can be seen that a precipitate of TMV with its specific antibody behaves in like manner with respect to the homologous antigen in excess amounts.

DISCUSSION

The strains of tobacco mosaic virus studied in this investigation closely resemble the parent type in their physical and chemical characteristics. The relatively minor differences which do exist, however, with respect to their amino acid content are in the quantitative amounts of arginine and isoleucine present in the green aucuba strain and the differences in the percentage of glutamic acid and lysine in the J14D1 strain. The serological behavior of these strains is somewhat a reflection of these minor changes in chemical composition in spite of the marked differences between them with respect to their biological activity. Serological cross reactions were demonstrated which were complete as far as could be determined by the quantitative precipitin methods used. Independent antigenic determinants of minor character were found to exist when absorptive technics were applied. Undoubtedly, these independent determinants could be identified by refinement of the quantitative procedures.

Considering the common characteristics of tobacco mosaic virus and its strains there must exist a peculiarity in composition, as perhaps a structural similarity, which is responsible for their basic properties. It is permissible, perhaps, to regard the active patches which determine the specificity for tobacco mosaic virus as being similar if not identical to those which determine the specificity for the green aucuba and the J14D1 strains with respect to the surface amino acid groupings. The assumption which seems somewhat compatible with the serological findings is that these minor differences in amino acid composition are only of secondary importance in determining the serological specificity since they perhaps must act at an appreciable distance from the specific directing groups.

It is of interest to note that the green aucuba and the J14D1 strains differ relatively little serologically from tobacco mosaic virus as compared to the Holmes' rib-grass strain (11) which differs markedly from the parent type in amino acid composition and especially with regard to its tyrosine and histidine content. The possibility that aromatic groups may be of greater importance than others for certain proteins in determining specificity was indicated by Kabat and Heidelberger (23) and Heidelberger (24). Further experimental work may perhaps disclose major difference in portions of the virus particle, other than the protein moiety, which may have some part in directing serological activity. For certain proteins a carbohydrate hapten plays a determinant part in respect to

specificity (25). Other types of proteins are known to contain characteristic groups, such as the phosphoric acid in casein, which may account for its specificity to a certain degree. Nucleic acids have been found to react specifically with certain antisera (26) and may exhibit a directive influence. Indeed, it has been variously reported that the nucleic-acid-free protein derived from tobacco mosaic virus reacts equally as well with anti-tobacco-mosaic-virus serum (27, 28) or not at all (29).

No logical explanation can be offered for the results obtained with the partially absorbed serum. The addition of a further amount of the absorbing antigen was seen to cause greater precipitation than did the addition either of the homologous or a second heterologous antigen. So far as is known, such a phenomenon has not been previously described.

It was desirable to determine whether the virus of the antibody-antigen complex had altered the specificity of the antibody with which it was bound so that only virus homologous to the complex could be added. These experiments were carried out since the possibility existed that, after removal of the complex, either there still remained a quantity of the antibody-antigen complex in solution with specifically altered antibody or the remaining antibody had been altered by having been in contact with the absorbing antigen. However, it was seen that heterologous antigen would combine with the washed complex. The behavior was the same as observed for other protein-antibody systems in that antigen added in large excess caused the complex to become soluble (30). No further interpretations can be applied to this peculiar observation.

SUMMARY

Independent serological determinants are demonstrated by absorptive technics for the green aucuba and J14D1 strains of tobacco mosaic virus even though they are not apparent by the quantitative precipitin reaction. The implications of these findings are discussed.

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STUDIES ON THE ORIGIN AND DEVELOPMENT OF PLANT TERATOMAS INCITED BY THE CROWN-GALL BACTERIUM*

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Somewhat more than 30 years ago Magnus (1915) and Smith (1916) independently announced the discovery of a highly complex type of crown-gall tumor that contained, in addition to tumor cells, tissues and organs that showed a high degree of cellular differentiation and morphological structure. Smith (1917) believed that these atypical (embryonal) teratoid tumors or embryomas, as he called them, arose from groups of normal pluripotent cells that had been disrupted and set growing by the expanding tumor. Levin and Levine (1918, 1920) maintained, on the other hand, that the differentiated tissues arose as part of the new growth of the tumor and not from the normal cells of the host. Levine (1919) further called in question Smith's interpretation by asserting that the crown-gall tumor does not stimulate the pluripotent cells in the leaves of *Bryophyllum calycinum* where they occur in large numbers but rather inhibits their development. Smith (1921) answered the criticisms leveled against his work and reiterated the belief that a distinct morphogenetic stimulus exists in crown-gall tumors.

Levine (1923) distinguished two distinct types of crown galls on tobacco in addition to the common globular type. These he listed as the differentiated globular type which formed small leaf-like structures over its surface and which were believed to be the result of the differentiation of tumor tissue, and the axillary crown gall which consisted of the crown-gall tumor and a poorly developed axillary bud. He thought that the bud grew not because of the presence of crown-gall bacteria but due to a tumor-induced mechanical interference with the food supply.

More recently Locke *et al.* (1938) studied the development of adventitious buds on decapitated tomato stems that had been inoculated with attenuated and virulent cultures of the crown-gall bacterium. These studies demonstrated that plants inoculated with the attenuated culture developed leaves and shoots and, later, masses of gall tissue. From the description given it would appear that the leafy structures in this instance arose from normal tissue and not from the tumor. Braun and Laskaris (1942) described a leafy gall that had been initiated on decapitated tomato plants by an attenuated culture. Tumors arose that appeared to be similar to Levine's first type of leafy crown gall in that the surface of the tumor contained many small leaflets, most of which did not develop appreciably.

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As a result of recent work many of the older concepts of tumor formation have had to be revised. These studies demonstrated (White and Braun, 1942; Braun and White, 1943) that normal plant cells may be changed to permanently altered tumor cells. Continued investigations (Braun, 1943, 1947) showed, furthermore, that the bacteria are capable of accomplishing this cellular alteration in very short periods of time. Once the transformation has been fully consummated the tumor cells continue to develop independently of the bacteria. Some of these tumor tissues have now been maintained in culture for more than 5 years and they have not at any time during that period shown the slightest tendency to differentiate and to produce complex tissues or organs. When the bacteria-free tumor tissues are reimplanted into a healthy host of the same species from which they were derived, they again give rise to typical crown-gall tumors. In view of these findings it seemed desirable to reinvestigate the question of the origin and development of the highly complex types of crown-gall tumors. It is with this problem that the present paper is concerned.

Experimental Methods and Results.—All experiments reported here were carried out on the plant species *Kalanchoe daigremontiana* (Hamet *et* Perrier). This host appeared more suitable in many respects for the study of the origin and development of the structurally complex tumors than were plants previously used for this purpose.

While conducting experiments on this species it was observed that when the T37 walnut strain of the crown-gall bacterium was inoculated at different points along the stem, typical undifferentiated tumors were initiated. As the tumors expanded those that originated in the younger portions of the stem produced with considerable regularity structural elements that showed varying degrees of morphological differentiation. The tumors that developed on the older more highly differentiated internodes continued, for the most part, to develop as typical undifferentiated crown-gall tumors. Other strains of the crown-gall bacterium (B2, B6), which initiate the formation of larger tumors on most susceptible plants including *Kalanchoe* than did the T37 strain,¹ produced these complex tumors far less regularly or not at all. There are, then, pronounced differences in the ability of the various strains of crown-gall bacteria to initiate the production of these structures. The two types of response are shown in fig. 1.

Description of Teratomas.—The morphologically complex structures found to arise on the tumors consisted mostly of leaves and stems. Many of the roots that arose from, or in the proximity to the tumor originated from the deeper healthy tissue. We have, however, on occasion observed crown-gall tumors on *Kalanchoe* that were composed almost entirely of highly distorted, greatly

¹ The author is deeply indebted to Professors George L. McNew and A. J. Riker for kindly supplying the B strains and the T37 walnut strains of the crown-gall bacterium used in this study.

thickened, brown root-like structures which, instead of producing leaves and stems, gave rise to highly abnormal roots at their periphery. The tumors that gave rise to leaves and stems occurred far more commonly and it is with these that the present discussion is concerned. The leaves borne on the tumors



FIG. 1. *Kalanchoe* plants inoculated with (a) T37 walnut strain of the crown-gall bacterium, (b) the B2 strain. Note the T37 strain initiated the production of a complex tumor at the uppermost point of inoculation. All tumors produced by the B2 strain were of the undifferentiated type. (Photographs by J. A. Carlile.)

ranged in appearance from normal to some that were so highly distorted that they were recognizable only by histological examination. Some of them were normal green in color, others were brown, red and yellow. Certain of the leaves consisted of a single prong-like process while others were composed of a series of prongs similar to those described by Küster (1926) on the dandelion root. A delicate collar of tissue, which projected for a distance of $\frac{1}{2}$ mm. entirely around such a thin pointed leaf, was sometimes observed. Fused and twin leaves as well as dichotomously branched and asciate leaves frequently developed. A number of different types of malformed leaves are shown in fig. 2.

Other extremely abnormal structures were also observed, and taken together they presented a very grotesque morphological picture. Similar abnormalities have not been observed by the writer to develop from a normal plant.

Origin of the Complex Tumors.—The fundamental problem involved here is concerned with the question as to whether the morphologically complex structures that develop on the tumors arise from normal cells, or whether the tumor cells themselves are capable under certain conditions of undergoing complex histological and morphological differentiation. It was necessary to eliminate,

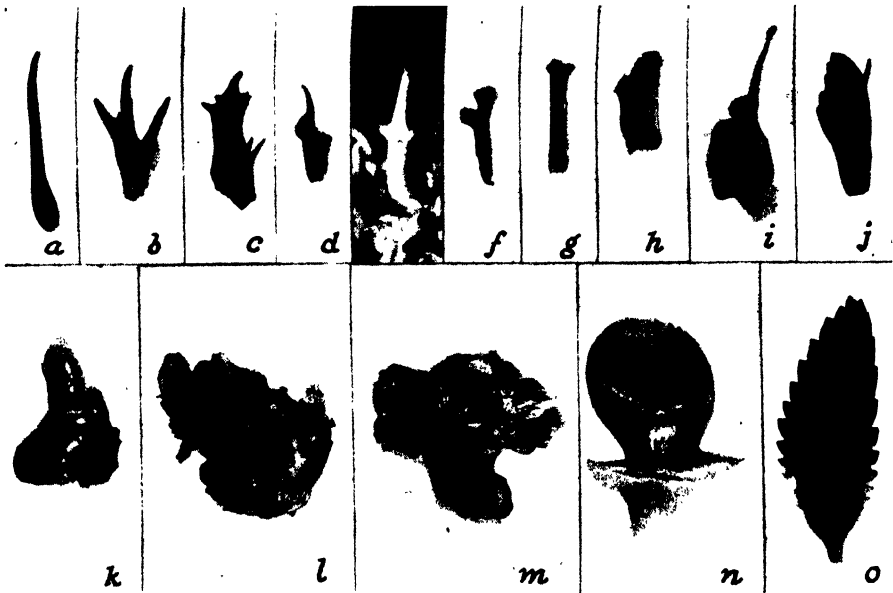


FIG. 2. Morphologically malformed leaves of the type that developed from the complex tumors. (Photographs by J. A. Carlile.)

as far as possible, the likelihood that normal plant cells were being carried along with the expanding tumor. Smith (1921), for example, has pointed out the fact that when a crown gall develops under the normal cortex the cortical cells may be lifted up and may grow with the growth of the tumor without actually being part of the tumor tissue. In order to minimize the possibility of normal cells being carried with the developing tumor, decapitated *Kalanchoe* plants were used. A single cut with a sharp razor was made through a young internode of the stem. It was observed that the cells immediately below the cut surface die very early and that it is from the cells just below these that the tumor appears to arise. Since most plant cells are held rigidly in place one would expect the tumor cells to outdistance normal cells in tumors originating in this manner. Fig. 3 shows two early stages in the development of such a tumor.



FIG. 3. Two stages in the development of a complex tumor that originated at the cut stem surface of a decapitated *Kalanchoe* plant; (a) 18 days after inoculation with the T37 strain. The tumor at this stage is of the undifferentiated type. (b) The same tumor 5½ weeks after inoculation. (Photographs by J. A. Carille.)

It is evident from these pictures that the tumor unquestionably develops first and the morphologically complex structures arise as a secondary process.

Histological sections were made of the material described above as well as of tumors that developed on the leaves of *Kalanchoe*. Leaf tumors were used in this study because, if the veins are avoided in making the bacterial inoculation, the tumors then originate from relatively few layers of histologically similar cells. Here again the tumors developed first and the complex differentiated structures appeared secondarily, as shown in fig. 4. Histological sections through tumors of the type shown in fig. 4b as well as sections through tumors that arose on decapitated stems demonstrated that it is the cells near or at the periphery of the tumor and consequently those farthest removed from the point



FIG. 4. Three stages in the development of complex tumors that originated in leaves of *Kalanchoe*. The tumor developed first and the morphologically complex structures arose secondarily. (Photographs by J. A. Carlile.)

of inception that differentiate. Fig. 5 shows a number of stages in the development of the leafy structures. The cells from which the teratological elements arose in this tumor were very small hyperplastic cells typical of those found in a rapidly expanding tumor. The abnormal cellular composition of the leaves is also well illustrated in fig. 5. While the histological picture strongly indicates that it is the tumor cells themselves that differentiate and produce morphologically complex structures, it is not possible at the present time to be absolutely certain that normal plant cells may not have been carried along with the growing tumor. Histological methods have not yet been devised to differentiate between tumor cells and stimulated normal cells.

The Inoculation of Normal and Teratological Structures with Bacteria.—Further studies were conducted in an attempt to resolve the question as to whether normal plant cells had been stimulated to growth and differentiation by the expanding tumor, or whether the tumor cells had recovered in varying degrees from the effects of the tumor-inducing principle. The teratological and

normal-appearing structures found on the tumor were therefore inoculated with the crown-gall bacteria. It was believed that if these structures were composed of tumor tissue they would not be susceptible to reinoculation with the crown-gall bacteria, because it was reasoned that cells already altered to tumor cells could not again be changed in the same direction. It should be recalled that all living cells of a susceptible host, with few exceptions, are capable of being transformed to tumor cells by the crown-gall bacteria. The results of many such inoculations demonstrated that the vast majority of the more or less abnormal leaves did not respond to inoculation with the formation of tumors. The results of one typical inoculation are shown in fig. 6. The inoculated leaf in this instance was round and fleshy throughout the greater

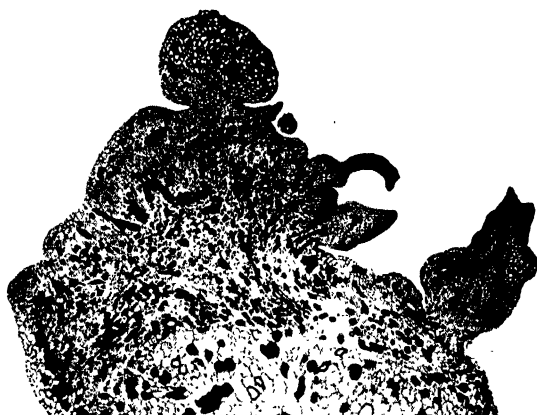


FIG. 5. Histological section through a tumor of the type shown in fig. 4,b. The origin and stages of development of the teratological structures are shown. (Photograph by J. A. Carlile.)

part of its length and highly abnormal in appearance. The picture was taken 2 months after the bacteria were introduced into the leaf and results of the inoculation are completely negative. Histologically this fleshy structure was composed essentially of leaf parenchyma cells which contained many chloroplasts. When normal leaf tissue containing histologically similar cells of comparable age were inoculated with the bacteria, tumors developed promptly.

When, on the other hand, the normal-appearing structures found on the tumors were similarly inoculated, tumors frequently appeared at the points of inoculation. Individual leaves sometimes showed varying degrees of response, some portions producing large tumors, others small ones or none at all. The results demonstrate, therefore, that inoculation of these structures results in all gradations of response ranging from unquestionable tumor formation in the normal-appearing structures to completely negative results in the morpho-

logically abnormal ones. Here again, however, although the results are suggestive, they are not entirely conclusive. Very little is as yet known of the factors involved in tumor inception and development and interpretations other than the one suggested above might therefore explain the inability of the abnormal structures to respond to inoculation with the crown-gall bacteria.

Growth Substances.—It is well known (de Ropp, 1947) that crown-gall tumor tissue generates a growth substance that behaves physiologically like certain of the known synthetic growth hormones. Locke *et al.* (1939) have demonstrated the presence of what appeared to be indol acetic acid in crown-gall tumors. It

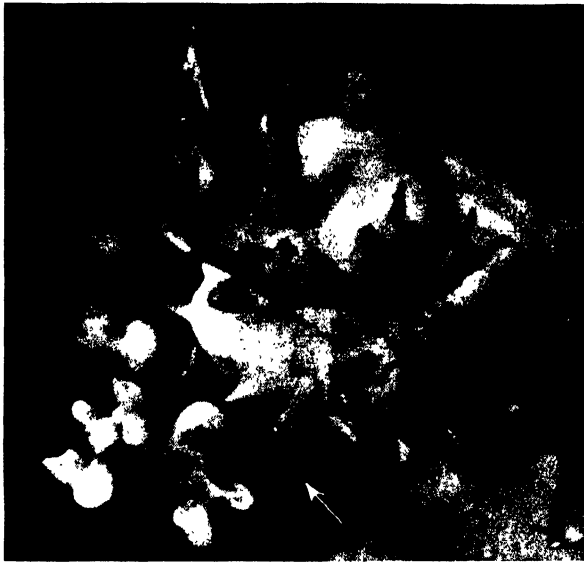


FIG. 6. Result of inoculation with the T37 strain of a highly malformed leaf. The great majority of abnormal leaves of the type shown here did not respond with the formation of tumors to inoculation with the crown-gall bacteria. (Photograph by J. A. Carlile.)

was therefore of interest to determine whether representative substances of this type were capable of initiating the production of leafy structures comparable to those produced by certain strains of the crown-gall bacterium on *Kalanchoe*. Indol acetic acid and naphthalene acetic acid were used in these experiments. These substances were applied in lanolin to shallow V-shaped notches cut into the various internodes of the stem. Concentrations of the active substances ranged in ten-fold increases from 0.0001 mg. per gram of lanolin to 10 mg. per gram of lanolin. In some experiments a single application was administered, while in others three separate applications were made at 10-day intervals for a period of 30 days. The higher concentrations of both these substances caused

the initiation and production of large numbers of roots, while concentrations of 0.1 mg. to 0.001 mg. per gram of lanolin stimulated the formation of gall-like overgrowths. However, leafy structures similar to those produced in the younger portions of the stem by certain strains of the crown-gall bacterium were not observed in any case.

In other experiments the concentrations of the two growth substances used above were applied to the cut surfaces of young decapitated *Kalanchoe* plants. The plants responded in these experiments as did the plants in the experiments described above. Root formation was stimulated at the higher concentrations and gall formation at the lower ones. It was observed, however, that adventitious buds and leaves developed at the cut surfaces of some of the plants. The buds were first observed at about the same time that the axillary buds began to develop. On occasion the adventitious buds assumed dominance and developed into normal shoots. For the most part, however, the axillary buds were dominant and the adventitious growths remained small. Usually, if present at all, only one adventitious bud or leaf was formed on a plant, and they did not show the morphological abnormalities found on the bacteria-incited teratomas.

The possibility remained, however, that a substance was produced by the expanding tumor that possessed greater formative properties than did the chemicals tested here. To study this possibility several experimental procedures were used. According to the first method *Kalanchoe* plants were decapitated in the usual manner and the cut surface was permitted to heal for 2 days. V-shaped cuts were made parallel to and 2–3 mm. below the cut surface on the four sides of the stem. Four 6 hrs. later crown-gall bacteria of the T37 strain were carefully introduced into the wounded areas. A period of several hours was permitted to elapse between wounding and inoculation in order to enable the water soaked area around the wounded tissue to dry and thus keep the bacteria confined to the area of the wound. Despite the fact that complex tumors developed just below the cut surfaces of the *Kalanchoe* stems, the normal cells at the cut surfaces were not stimulated to develop into either normal or abnormal structures even though, as the tumors expanded, these cut surfaces appeared to become part of the external surface of the new growth.

The second method used to determine whether formative growth substances were generated by tumor tissue was one in which large pieces of sterile sunflower tumor tissue were placed on the cut surfaces of decapitated *Kalanchoe* plants and permitted to develop there. The tissues were first grown in sterile short-necked funnel-shaped glass tubes that were plugged with cotton at the top and at the neck. The tissue was fed by placing White's medium (White, 1943) around the tissue, the top and bottom of the tissue were free of agar. Stems of *Kalanchoe* plants were then sterilized with alcohol and mercuric chloride, decapitated, and the cut stem surface inserted through the neck of the sterile

system and placed in intimate contact with the tumor tissue. Sterile cotton was wrapped around the upper portion of the stem and the neck of the funnel in order to keep the system sterile. Despite precautions to maintain sterility it was not possible in most instances to keep the system sterile for more than 3 weeks. However, the tumor tissues did not during that period initiate the formation of adventitious buds or teratological structures at the cut stem surface of the *Kalanchoe* plants.

Kalanchoe daigremontiana is an unusual plant species in that it characteristically produces small plantlets (fig. 7) at the edges of its leaves. When these plantlets mature they drop from the leaves and, if environmental conditions are favorable, they develop into plants characteristic of the species. It is interesting to note (fig. 8) that many of the leaves that develop from the tumor also produce or attempt to produce plantlets. These small plants ranged in appearance from mostly undifferentiated bulb-like processes to normal-appearing



FIG. 7. Normal leaf of *Kalanchoe daigremontiana* showing the development of plantlets that are characteristically produced at the edges of the leaves in this species. (Photograph by J. A. Carlile.)

structures. Plantlets borne on the same leaf were frequently very different in size and appearance. Certain of them were covered with multitudes of minute knobs, others had prong-like processes similar to those described earlier. In still others the leaves were very fleshy as compared to the leaves of normal plantlets.

If these plantlets were composed of normal tissue but were behaving abnormally because of their close association with the tumor tissue, it was believed that they would again revert to the normal type if they were removed from the influence of the tumor. It was observed early in these experiments, however, that when the majority of the more or less abnormal plantlets were removed from the tumor and placed on moist peat or soil, they did not produce roots, and hence failed to develop to any appreciable extent. Plantlets that develop on the leaves of normal plants, on the other hand, always produce a root system under these conditions and develop into plants typical of the species. Because the majority of abnormal plantlets that developed from teratological leaves did not produce roots, the problem was approached in part with the use of tissue culture methods. More than fifty of these plantlets were washed in

sterile tap water under sterile conditions for about 24 hrs. and they were then placed on White's medium (White, 1943) and permitted to develop there. Some flasks of media contained no added growth-promoting substance, while others containing indol acetic acid in concentrations of 10, 1 and 0.1 mg. per liter of media. The results of this study showed that, despite the fact that certain flasks of media contained a root-inducing substance in varying concentrations, the vast majority of the plantlets failed to develop roots and made only slight growth. *Kalanchoe* plantlets obtained from leaves of normal plants readily developed roots under these conditions. It appears likely that the more

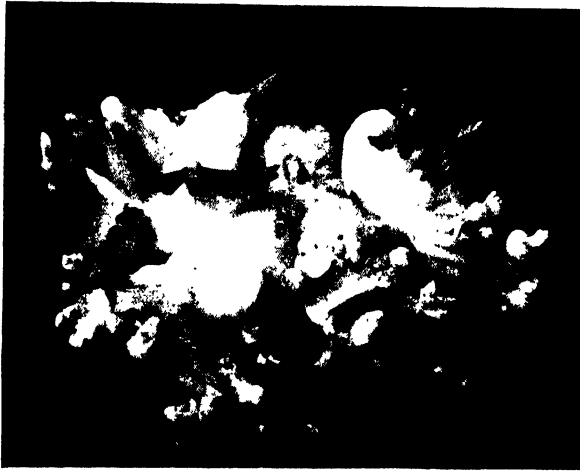


FIG. 8. The production or attempted production of plantlets on leaves that originated from a complex tumor. The plantlets ranged in appearance from bulb-like processes to normal-appearing structures. Compare with fig. 7. (Photograph by J. A. Carlile.)

normal of the plantlets, such as those that developed a well formed apical bud, would develop typically if they could be made to produce a root system or if successfully grafted to a healthy plant. A few of the more abnormal plantlets obtained from the teratomas, on the other hand, developed white wart-like growths on their leaves and after continued incubation these developed into masses of slow-growing callus-like tissue that finally completely overgrew the plantlet itself (fig. 9). Dedifferentiation had occurred in part and the plantlet apparently lost partial control of the cells under these cultural conditions. This gall-like tissue has now been carried through four transfers over a period of 8 months. In contrast, it is interesting to note that normal *Kalanchoe* tissue isolated from the region of the cambium failed to make appreciable growth on this culture medium.



FIG. 9. Mass of slow-growing gall-like tissue that developed from a leaf of an abnormal plantlet that had been grown in tissue culture. Cellular dedifferentiation had apparently occurred and the plantlet was completely overgrown by the mass of tissue. (Photograph by J. A. Carlile.)

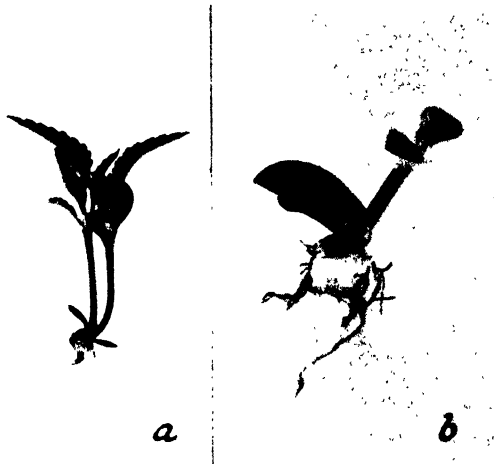


FIG. 10. Two plantlets that originated on the leaves of a teratoma. These plantlets produced a sterile gall-like overgrowth in place of a normal root system. (Photographs by J. A. Carlile.)

The entirely normal-appearing plantlets found on leaves of the teratomas were placed on soil or moist peat and permitted to develop there. A few of these apparently normal plantlets, instead of developing a root system, produced masses of gall tissue at their base (fig. 10). The gall tissue in each case

was subjected to prolonged washing with sterile tap water and the entire mass triturated in a sterile mortar. Brain-heart infusion agar (Difco) was used in pouring the plates. The masses of gall tissue were found to be free of the crown-gall bacterium. Several possibilities present themselves in interpreting these findings. The crown-gall bacteria may have initiated the gall-like overgrowths but may have died before isolation attempts were made. This seems rather unlikely in view of the fact that bacterial isolations made from tumors known to contain the bacteria showed that the organisms persisted in the tissues of this plant in large numbers for long periods of time. Tumor cells may have been present, on the other hand, at the point of origin of the overgrowth and the gall may have developed from these. It is also possible that dedifferentiation had occurred in the cells as it apparently did in culture, giving rise to the overgrowth.

Other of the normal-appearing plantlets found on leaves that originated in the complex tumors produced a normal root system and developed into what appeared to be typical *Kalanchoe* plants (fig. 11). There is found, then, an almost continuous series of morphological developments ranging from highly malformed, mostly undifferentiated processes to what appear to be normal plants.

The morphological differences observed in these structures could possibly be explained on the basis of the presence of a mixture of tumor and normal cells in the same structure. The highly malformed growths would, according to this concept, be composed predominantly of tumor cells but would also contain normal cells. The normal structures, on the other hand, would consist entirely of normal cells that had never been altered by the crown-gall bacteria.

If this is postulated, however, it must be assumed that normal cells are present in large numbers at the periphery of the tumor. Present methods, unfortunately, do not permit us to determine whether this is true. Nevertheless, because of the great growth capacity shown by tumor cells, it is believed that at least those portions of the tumor farthest removed from the point of inception are composed entirely of tumor cells. This would appear to be particularly true in the tumors that originate at the cut stem surfaces of decapitated plants. The multiplication of tumor cells in these instances is unhindered by overlying normal tissue. As has already been noted it is only at the extreme periphery of such tumors that the teratological and normal structures originate. It is suggested, therefore, that the development of the malformed as well as the normal-appearing structures present in the complex tumors might well be explained on the basis of the partial or complete recovery of the tumor cells from the effects of the tumor-inducing principle.

Discussion.—The fundamental question as to whether the morphologically complex structures that develop from certain crown-gall tumors are the result of the growth of normal host cells that have been stimulated by the expanding tumor as maintained by Smith, or whether the tumor cells themselves are



FIG. 11. An apparently normal plant that developed from a plantlet that originated on the leaves of a complex tumor. (Photograph by J. A. Carlile.)

capable under certain conditions of undergoing complex histological and morphological differentiation as contended by Levin and Levine has been

investigated. The results reported here are difficult to interpret and they are admittedly not entirely conclusive. The writer does not believe, moreover, that they can be conclusive until cytological or other suitable methods have been devised that will differentiate with certainty between altered tumor cells and normal cells. These two cell types can, of course, be readily distinguished biologically by implanting bacteria-free fragments of tissue composed of either tumor or normal cells into a healthy plant of the same species from which they were originally derived. Under these conditions the tumor fragments give rise to typical crown-gall tumors, whereas the normal fragments fuse with the host and soon fall into the normal growth pattern of the host.

The evidence obtained from the several different types of experiments reported in this study suggests, however, that the morphologically complex structures found in the teratomas are the result of the growth of tumor cells that have recovered in varying degrees from the effects of the tumor-inducing principle. A consideration of the results based on this interpretation and discussed in the light of recent findings dealing with the cellular alteration in the crown-gall disease is, therefore, tentatively presented.

In interpreting the results according to this concept two fundamental factors must be considered. The first of these is concerned with the tumor-inducing principle associated with the cellular conversion, while the second deals with the relative competence of the host cells upon which this factor acts.

As indicated earlier, when the tumor-inducing principle acts on certain plant cells it initiates tumors whose cells possess only very limited powers of differentiation and whose growth is completely autonomous in the host as well as in culture. When presumably this same tumor-inducing principle acts on *young cells* of certain plants such as *Kalanchoe* it also initiates tumors whose cells appear to possess at first only limited powers of differentiation. As the tumor expands, however, partial or complete recovery of some of these cells seems to take place giving rise to a chaotic assembly of structures that show varying degrees of morphological differentiation. The impression gained, therefore, is that the type and competence of the cells acted upon, and perhaps the reactivity of the tissues in which the cells are embedded, are of fundamental importance in determining the type of structure that ultimately develops.

There appears to be general agreement among biologists that the locus of cellular differentiation is in the cytoplasm of the cell. The character of animal cells is often specifically determined very early in their development, while the somatic cells of higher plants may and often do remain pluripotent. This property of cellular pluripotency varies greatly not only in different plant species but also in different cells of the same individual of a given species. Bloch (1947) has suggested, on the basis of his studies on certain specialized cells in *Ricinus*, that the behavior of those plant cells involves a change in their cyto-

plasmic system in which control exerted by determinants in the nature of plasmagenes may play a rôle.

The results described here can perhaps be interpreted by assuming the presence in plant cells of factors that determine the morphogenetic fate of the cells. In those instances in which undifferentiated tumors develop as a result of bacterial inoculation the factors concerned with morphogenetic determination must be assumed to be completely overwhelmed by the action of the tumor-inducing principle. The latter appears capable of cancelling in large part the various competences for differentiation previously possessed by the affected cells. As evidence of this, tissue cultures of tumors isolated from sunflower and *Vinca rosea* have now been kept in the laboratory for more than 5 years without showing any evidence of a tendency to differentiate and produce complex tissues and organs. When, on the other hand, this same tumor-inducing principle acts on *young cells* of plants such as *Kalanchoe*, the balance must be assumed to be in favor in varying degrees of the morphogenetic determiners present in the affected cells. The result of such a condition would probably be the development of structural elements that show varying degrees of differentiation such as have been reported here. If this interpretation is correct, then it is of interest to note that the potentialities for differentiation in certain young cells appears to be so great that complete recovery of affected cells takes place and apparently normal plants are produced.

SUMMARY

When the T37 walnut strain of the crown-gall bacterium was inoculated into *Kalanchoe* plants at different points along the stem typical undifferentiated tumors similar to those found on many other hosts were produced. As the tumors enlarged those that originated in the younger portions of the stem produced leaves and stems that showed varying degrees of morphological differentiation. Many of these structures were highly distorted while others appeared more or less normal. Several types of experiments were designed to determine whether the morphologically complex structures that developed from the tumors were the result of the growth of normal host cells that had been stimulated by the expanding tumor or whether the tumor cells themselves were capable under certain conditions of undergoing complex histological and morphological differentiation. The results of these experiments, although not entirely conclusive, suggested that the morphologically complex structures were the result of the growth of tumor cells that had recovered in varying degrees from the effects of the tumor-inducing principle. A consideration of the results based on this interpretation and discussed in the light of recent findings dealing with the cellular alteration in the crown-gall disease is presented.

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PROPAGATION OF RABBIT FIBROMA VIRUS IN THE EMBRYONATED EGG*

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In view of the theoretical interest attached to the relationship of viruses to neoplasms,¹ it seems worth while to report the adaptation to growth in the embryonated egg of a virus which, if it does not cause a true tumor, is at least the etiologic agent of a closely related tumor-like condition. Adaptation of such a virus to a different and relatively simple experimental host such as the developing chick embryo may provide a means of studying the virus-tumor relationship. In fact the earliest use of the chick embryo in the study of viruses was for just this purpose, when Murphy and Rous obtained growth of a fowl sarcoma on inoculating into the chick embryo either minced tumor tissue or a Berkefeld filtrate of such tissue.² The virus studied here is that of rabbit fibroma isolated by Shope³ from a growth on the foot of a wild rabbit. Passed since then in series through domestic rabbits, one strain (the so-called OA strain) has maintained its ability to produce tumors on subcutaneous or intratesticular inoculation. From this original strain there arose a variant (the so-called IA strain described by Andrewes⁴) which had lost the ability to produce tumors and caused only inflammation.

After many unsuccessful attempts at growing strains of the fibroma virus in tissue culture, Faulkner and Andrewes⁵ finally succeeded in adapting the IA strain to growth in cultures of rabbit testis.

Paschen,⁶ was unable to maintain the IA strain on the chorioallantoic membrane for more than one passage, although the OA strain was still present after 5 passages. However methods and findings are not given in any detail.

Experimental. Methods. The strain of virus employed in most of our experiments was the OA, or tumor-producing strain, obtained from Dr. R. E. Shope as glycerinated rabbit testicle. The IA strain was used similarly in the form of glycerinated rabbit testicle. *Titrations* were carried out by grinding a weighed piece of tissue with sand in a mortar and adding sufficient cold veal

* Part of work reported here was carried out while the author was a National Research Council Fellow in the Medical Sciences.

¹ Rous, P., *Bull. New York Acad. Med.*, 1947, **23**, 65.

² Murphy, J. B., and Rous, P., *J. Exp. Med.*, 1912, **15**, 119.

³ Shope, R. E., *J. Exp. Med.*, 1932, **56**, 793.

⁴ Andrewes, C. H., *J. Exp. Med.*, 1936, **63**, 157.

⁵ Faulkner, G. H., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1935, **16**, 271.

⁶ Paschen, E., *Zent. Bakt.*, 1936, **138**, 1.

infusion broth or saline to make a 10% suspension. Further tenfold dilutions were then made in cold broth, and inoculated subcutaneously in a rabbit in 1.0 cc amounts, no more than 9 tests being carried out in any one animal. The rabbits were observed for appearance of subcutaneous tumors at the site of inoculation. *Neutralization* tests were carried out according to Shope's⁷ method, *i.e.* a mixture of 1 cc of serum and 1 cc of infection suspension was incubated at 37°C for 1 hour, stored for 2 hours in the refrigerator, then injected in 1 cc amounts into the subcutaneous tissue of a rabbit, together with appropriate controls in the same rabbit.

Results. Adaptation of the Virus to Eggs. The stored glycerinated testicle virus was well washed in cold saline, then ground with sand and saline to make a heavy suspension, which was used to infect a fresh rabbit, 0.5 cc being inoculated into each testicle. Seven days later, when scrotal and testicular edema were pronounced, the animal was killed, small pieces of testicle were ground with sand and sufficient cold saline to make a 1% suspension by weight. After this suspension had been allowed to settle for about an hour in the refrigerator, 0.1 cc of the suspension was dropped onto the chorioallantoic membrane of 4 12-day-old eggs and was also cultured on a blood agar slant. Three days later, after incubation at 35°C, the membranes were harvested, ground lightly in a pyrex glass grinder, together with 4 cc of saline per membrane. After standing for half an hour the supernatant of this suspension was used as inoculum for a new set of eggs. Passages were made in this fashion at regular 3-day intervals. The membrane suspension from the 7th egg passage produced typical fibroma in a rabbit. The egg passage virus having unfortunately become contaminated at the 8th passage, the testicle from the rabbit injected with 7th passage egg membrane suspension was used as a source of virus. The virus was then maintained in eggs for 18 consecutive passages without mishap. In this second series membranes of the 1st, 5th, 10th, 14th, 15th and 18th passages were found to be infectious for rabbits.

Numerous attempts were made to adapt the virus to growth on the chorioallantoic membrane. In each case where virus from fresh tissues was employed it multiplied in the egg. However glycerinated virus did not always establish itself. In some cases the virus passage was discontinued after only 3 or 4 transfers, in others it was carried through 10 or 12 transfers. It was found that the size of the inoculum could be varied between 0.1 and 0.35 cc, the membranes could be ground in physiological saline, saline buffered with phosphate, veal infusion broth, or even egg fluid, though the latter was less satisfactory. It was, however, found necessary to make passages quite regularly at 3-day intervals. The titer of virus was lower on the 2nd and 4th days than on the 3rd, so that passage at 4-day intervals resulted in loss of the virus within 2 to 3 passages. Eggs which had been incubated for 11 days prior to

⁷ Shope, R. E., *J. Exp. Med.*, 1932, 56, 803.

inoculation sustained the growth of the virus most satisfactorily, regularly yielding membranes which were infectious for rabbits when diluted 10^{-4} or 10^{-5} by weight.[†] The titers obtained with 9-day eggs were slightly lower and the titers with 13-day eggs approximately 100-fold lower.

In later passages, whenever possible contamination was feared, penicillin (165 units per egg) and streptomycin (0.0015 g per egg) were added to the membrane suspension.

Viability of Egg Passage Virus. Suspensions of membranes made up in veal infusion broth pH 7.3 to which 20% rabbit serum was added were found to withstand quick freezing with dry ice and alcohol and storage in the dry ice chest for at least 2 months. Suspensions in saline, in egg fluid or even in plain broth were often inactive on thawing. Virus suspensions allowed to stand overnight in the refrigerator showed a roughly 100-fold fall in titer.

Distribution of Virus within the Egg. On several occasions the embryos, yolk sacs, and pooled egg fluids from infected eggs were tested for virus by subcutaneous inoculation into a rabbit. Even when the chorioallantoic membrane suspension proved infectious to rabbits when diluted 10^{-4} or 10^{-5} , no virus was detectable in any other part of the egg.

Routes of Inoculation. Virus which had been through a number of passages on the chorioallantoic membrane was injected in 0.5 cc amounts into the yolk sac. Embryos and yolk sacs harvested 3 to 5 days later contained no demonstrable virus.

Effect of Virus upon the Chick Embryo and its Membranes. In view of the widespread lesions produced on the chorioallantoic membrane by the related myxoma virus,^{8, 9} it was a disappointment to find no consistent and recognizable effect of fibroma virus on the chick embryo. Many membranes which were shown by rabbit inoculation to contain virus in large amounts appeared as delicate in structure as membranes from normal eggs. On the other hand it seemed, in the course of adapting virus from a number of individual rabbits to growth in the egg, as if the strains derived from some rabbits showed more tendency to cause marked edema of the membrane than did other strains. Passages were made in parallel from edematous and from "non-edematous" membranes. Sometimes the edema-producing capacity would remain for a passage or two before fading out. Once it had disappeared it would not reappear in the course of further passages. The nature of this "lesion" is being subjected to further study at the present time.

[†] Tumor tissue or testicle removed from a rabbit at the height of infection, i.e. about 6 days after inoculation, is usually infectious when diluted 10^{-5} or even 10^{-6} . Thus the titer obtained with egg passage virus even under optimal conditions is not quite so high as in the case of rabbit passage virus.

⁸ Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1937, 15, 131.

⁹ Haagen, E., and Du Dscheng-Hsing, *Zentr. Bakt.*, 1938-39, 143, 23.

Sections of a number of membranes were studied histologically. The spindle shaped cells with large nuclei characteristic of fibroma in the rabbit^{10, 11} could not be demonstrated in the egg. No inclusion bodies were seen. Contrary to the experience reported by Paschen⁶ we were unable to demonstrate elementary bodies with certainty on smears stained with Morosow's stain.

The embryos of eggs infected with fibroma virus appeared normal. Several batches of infected eggs were allowed to hatch. The chicks from these eggs were not tested for presence of virus; however, serum obtained from them at the age of 15 days after hatching failed to neutralize rabbit passage fibroma virus. This lack of detectable immunologic response may well be due to the well-known fact that immature animals respond poorly to antigenic stimuli.

Demonstration of Presence of Virus in the Egg. In the absence of characteristic lesions, the only means of recognizing the presence of virus was back-passage into the rabbit. Quicker methods of recognizing infection in the egg were tried unsuccessfully. The supernates of infected membrane suspensions, both unheated and heated for 40 minutes at 70°C¹² were tested for hemagglutination against red cells from the chicken, duck, mouse, guinea pig, rabbit, horse and sheep; but the membranes from infected eggs produced hemagglutination no more regularly than did those of the uninfected eggs used as controls. Precipitin tests and skin tests in rabbits, using as antigen the allantoic fluid of infected eggs yielded negative findings.

Identification of the Virus Obtained upon Egg Passage. That the virus passed in the egg was actually fibroma virus was shown in 3 ways.

(a) Rabbits inoculated on different occasions with egg passage virus were challenged 2 to 3 weeks later with glycerinated rabbit passage virus and to this they were found to be immune.

(b) A rabbit was immunized by subcutaneous and intratesticular inoculation of egg passage virus and another rabbit was similarly immunized with rabbit passage virus. Both were bled after 14 days and their respective sera used in a neutralization test against fresh rabbit passage virus. Both sera neutralized the virus, no lesion appearing where the mixture was inoculated, whereas areas injected with control mixtures of virus and normal rabbit and virus and saline displayed tumor formation.

(c) A rabbit injected with 8th egg passage fibroma virus was challenged 6 weeks later with a subcutaneous injection of rabbit passage myxoma virus. A control rabbit receiving the same inoculum was dead of myxoma in 10 days, whereas the rabbit which had previously been exposed to egg passage fibroma virus showed no signs of illness. In view of the regularity with which myxoma, virus causes death in normal rabbits it seems certain that the inoculation with

¹⁰ Hurst, E. W., *Australian J. Exp. Biol. and Med. Sc.*, 1938, 16, 205.

¹¹ Ahlstrom, C. G., *J. Path. and Bact.*, 1938, 48, 461.

¹² Mills, K. C., and Dochez, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, 57, 140.

egg passage fibroma virus had protected the rabbit against an otherwise fatal infection with myxoma, a phenomenon first described by Shope⁷ in connection with rabbit fibroma.

Growth of the IA Strain of Virus in the Egg. Only 2 experiments were carried out with the IA strain of virus. In both cases the virus was lost at the 4th passage, owing to bacterial contamination. No lesions were observed in the infected eggs. In the one case in which the virus titer was determined it was only 10^{-2} at the 3rd passage.

Discussion. The experiments described here show that at least one strain (the OA strain) of the virus of infectious fibroma of rabbits can readily be propagated on the chorioallantoic membranes of the developing hen's egg. Although the titers of virus obtained from infected membranes compared favorably with these from rabbit tumor tissue, the virus did not, in our experience, show any tendency to invade the embryo and was not demonstrable in the extra-embryonic fluids. Likewise when virus propagated for a number of passages on the chorioallantoic membrane was injected into the yolk sac it rapidly disappeared. It is possible that virus which had been subjected to a greater number of passages on the chorioallantoic membrane might display greater invasiveness for the egg.

Passage through the embryonated egg did not seem to alter the type of lesion for its original rabbit host. This finding was rather unexpected, in view of the fact that the virus did not produce in the egg the pathological changes which are characteristic in the rabbit, *i.e.* intense fibroblastic proliferation with production of tumor-like masses and, in the wild cottontail rabbit, very striking cytoplasmic inclusion bodies in the overlying epithelium. No such bodies could be found in epithelium covering the chorioallantoic membrane.

Summary. 1. The virus which is the causative agent of infectious fibroma, giving rise to tumor-like nodules in rabbits, was maintained by serial passage on the chorioallantoic membrane of the embryonated hen's egg for 18 consecutive passages.

2. The titer of virus in infected chorioallantoic tissue was slightly lower than the titer in rabbit tissues. It was maintained by using eggs incubated for 11 days prior to inoculation and making passages at 3-day intervals.

3. Invasion of the embryo and extra-embryonic fluids did not take place in eggs inoculated on the chorioallantoic membrane. Inoculation of the virus into the allantoic sac, the amniotic sac, the yolk sac and the embryo itself yielded negative results.

4. Although marked edema of the chorioallantoic membrane was noted in some of the infected, no pathognomonic lesion, gross or microscopic, was present, so that it was necessary in every instance to resort to back-passage into rabbits to demonstrate the presence of virus.

5. The type of lesion produced in rabbits by the virus was not significantly altered by repeated passage in hen's eggs.

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